Steroid pre-receptor signalling in human endometrium

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Thesis submitted for the degree of Doctor of Philosophy, April 2008.

Declaration

Except where due acknowledgement is made by reference the studies undertaken in this thesis were the unaided work of the author. The work described in this thesis has not been previously accepted for, or is currently being submitted in candidature for another degree.

In Chapters 3 and 5, I acknowledge the assistance of Mr Ian Swanston and colleagues, in the MRC Human Reproductive Sciences Unit, Edinburgh, who carried out all the radioimmunoassay to measure serum hormone levels for endometrial dating. In these same chapters I acknowledge Dr Alistair Williams, Department of Pathology, Royal Infirmary of Edinburgh, who carried out all the histological dating of samples.

In addition I must acknowledge Dr Mick Rae, University of Edinburgh for help and assistance with designing and executing the experiments detailed in Chapter 4.

Sarah McDonald

April 2008

Acknowledgements

Thanks must firstly go to my co-supervisors, Professors Hilary Critchley and Ian Mason. They have been an invaluable source of guidance, support and inspiration throughout the course of my studies. I truly could not have got this far without them.

Huge thanks are also due to all the members of the labs I've worked with, in particular Teresa Henderson and Lyndsey Boswell, but all members of the "Hilary Critchley" and "Ian Mason" research groups have helped, supported and taught me along the way. Thanks also to everyone in the labs for providing such a pleasant working environment.

As all the work herein required human tissue, I'd like to thank all the women who kindly donated tissues, and to the research nurses, Catherine Murray and Sharon McPherson, without whom this work would not have been possible.

Many friends deserve a thank you for listening and cheering me up throughout the toughest times.

Finally, and most importantly, thanks to my parents, Anne and Robert, who have been behind me every step of the way and supported me in more ways than any of us ever thought would be required.

Abstract

The human endometrium is a highly dynamic tissue that undergoes cyclical tissue injury and repair. The function of the endometrium across the menstrual cycle is tightly regulated by steroid hormones, both systemically and locally derived. Local steroid availability is modulated by a number of steroid-metabolising enzymes, particularly members of hydroxysteroid dehydrogenase (HSD) families. In addition, steroid hormone action is also governed by the availability of the appropriate cognate receptors.

Key steroids of the endometrium are estrogens, androgens, progestogens and glucocorticoids. These are modulated at a local level by a number of steroid metabolising enzymes, including 11βHSDs, 3βHSDs, 3αHSDs (AKR1Cs), and 17βHSDs.

As all these steroids are crucial for normal control of endometrial function, it is desirable to understand the enzymes regulating their availability and action across the menstrual cycle, in early pregnancy, and following clinical treatments. Thus, the aims of this study were to investigate the expression (quantitative real-time PCR) and immunolocalisation of steroid metabolising enzymes and receptors in human endometrium across the menstrual cycle, in first trimester decidua, and in two clinical situations – use of a levonorgestrel intra-uterine system (LNG-IUS) to control uterine bleeding; and following recombinant FSH and GnRH antagonist treatment as part of IVF/ICSI procedures. Additionally, modulation of one of these

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enzyme families, 11βHSDs, and corresponding receptors, GR and MR, was investigated *in vitro*.

This work has shown that 11βHSD-1 mRNA is present at highest levels in the menstrual phase of the cycle and in first trimester decidua, the times when an inflammatory response is evident. 11βHSD-2 mRNA and protein are present at all stages of the cycle, and also in first trimester decidua. GR mRNA and protein are highly expressed throughout the cycle. MR mRNA expression varies across the cycle in a pattern similar to progesterone secretion. 11βHSD-1 mRNA expression is increased in response to IL-1α and cortisol, and GR mRNA shows a similar trend. 11βHSD-2 and MR expression are not altered by IL-1α or cortisol. 3βHSD-1 mRNA has been shown to be present only in first trimester decidua; 3βHSD-2 is not detectable by these methods. Immunohistochemistry using an antibody which detects both 3βHSD-1 and -2 has shown low levels of protein in the tissues studied. AKR1C1-3 mRNAs are expressed throughout the menstrual cycle; all three enzymes are predominantly expressed in the secretory phase. AKR1C3 is localised to the glandular and surface epithelial cells, and vascular endothelium. AKR1C4 mRNA is not detectable in the endometrium at any stage of the menstrual cycle.

Expression of steroid-metabolising enzymes is perturbed in the endometrium of users of a LNG-IUS, and also following GnRH antagonist treatment for sub-fertility.

These studies have shown that the endometrium has the ability to precisely regulate its balance of steroid hormone availability at a local level, and that this balance may

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be altered following administration of exogenous steroids. Further functional studies such as knockout or knockdown of these enzymes would expand this knowledge and fully elucidate steroid hormone metabolism and pre-receptor signalling.

Presentations relating to this thesis

Endometrial Expression of 11β -Hydroxysteroid Dehydrogenases (11βHSDs) and the Glucocorticoid Receptor (GR) Across the Menstrual Cycle, in First Trimester Decidua and in Endometrium Exposed to Intra-uterine Levonorgestrel (LNG-IUS)

Oral communication, Munro Kerr Society, Edinburgh, April 2004

Expression of 11β-HSDs, GR and MR in human endometrium

Poster presentation, Scottish Society for Experimental Medicine, Edinburgh, November 2004

Androgen and estrogen metabolism in human endometrium

Oral communication, Munro Kerr Society, Edinburgh, March 2006

Endometrial intracrinology: Expression of 3β-hydroxysteroid dehydrogenase (3βHSD), and 17β-hydroxysteroid dehydrogenase type 5 (17βHSD-5)

Oral communication, European Congress of Endocrinology, Glasgow, April 2006

A 17βHSD "switch" in human endometrium

Poster presentation, ENDO 2006, Boston, USA, June 2006

Peer-reviewed publications

McDonald SE, Henderson TA, Critchley HOD, Mason JI, 2006. "**11beta-hydroxysteroid dehydrogenases in human endometrium**", Mol Cell Endocrinol. 248(1-2):72-78.

Vani S, McDonald SE, Williams ARW, Mason JI, Thong KJ, Critchley HOD, 2007 "**Midluteal endometrial intracrinology following controlled ovarian hyperstimulation and use of a gonadotrophin releasing hormone antagonist.**" Hum Reprod 22(11):2981-2991.

Copies of these papers can be found in Appendix 2.

Contents

List of figures

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Abbreviations

+ve	positive
-ve	negative
β-Me	beta-mercaptoethanol
11βHSD	11beta-hydroxysteroid dehydrogenase
17βHSD	17beta-hydroxysteroid dehydrogenase
20αDOC	20alpha-deoxycortisol
20αOHP	20alpha-hydroxyprogesterone
3αHSD	3alpha-hydroxysteroid dehydrogenase
3βHSD	3beta-hydroxysteroid dehydrogenase
ABC-HRP	avidin-biotin complex-horseradish peroxidase
AKR	aldo-keto reductase
AME	apparent mineralocorticoid excess
AMPS	ammonium persulfate solution
AR	androgen receptor
BTB	breakthrough bleeding
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
COX-1	cyclo-oxengenase-1
D	decidua
DAB	3,3-diaminobenzidine
DHEA	dehydroepiandroserone
DHT	dihydrotestosterone
DMSO	Dimethyl sulphoxide
DOC	deoxycortisol
E_2	estradiol
EB	endometrial biopsy
ECL	enhanced chemiluminesence
EDTA	ethylenediamine tetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	estrogen receptor
ES	early secretory
FSH	follicle stimulating hormone
GnRH	gonadotrophin releasing hormone
GR	glucocorticoid receptor
H&E	haematoxylin and eosin
hCG	human chorionic gonadotrophin
HOSE	human ovarian surface epithelium
HPA (axis)	hypothalamic-pituitary-adrenal
HPG (axis)	hypothalamic-pituitary-gonadal
HSD	hydroxysteroid dehydrogenase
ICSI	intra-cytoplasmic sperm injection
Ig	immunoglobulin
IGFBP-1	insulin like growth factor binding protein-1

IHC	immunohistochemistry
IL-1	interleukin-1
IL-1R	interleukin-1 receptor
IVF	in vitro fertilisation
JAK/STAT	Janus kinase/ Signal Transducers and Activators of Transcription
JNK	Jun N-terminal Kinase
kDa	kilodaltons
LGL	large granular lymphocyte
LH	luteinising hormone
LMP	last menstrual period
LNG	levonorgestrel
LNG-IUS	levonorgestrel releasing intrauterine system
LS	late secretory
M	menstrual
MMP	matrix metalloproteinases
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
MS	mid secretory
n/a	not applicable
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (hydrogenated)
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (hydrogenated)
NBF	neutral buffered formalin
OR	oocyte retrieval
OSE	ovarian surface epithelium
P	proliferative
P_4	progesterone
PBS	phosphate buffered saline
PBST	phosphate buffered saline + Tween 20
PCR	polymerase chain reaction
PGD	prostaglandin D
PGF	prostaglandin F
PR	progesterone receptor
PRKO	progesterone receptor knockout
PVDF	polyvinylidene difluoride
QRT-PCR	quantitative real-time PCR
rFSH	recombinant follicle stimulating hormone
RIA	radioimmunoassay
RIN	RNA integrity number
RNA	ribonucleic acid
RNAi	RNA interference
RPMI	Roslin Park Memorial Institute
RT-PCR	reverse-transcriptase PCR
RU486	mifepristone
scc	side-chain cleavage (enzyme)
SDR	short-chain dehydrogenase/reductase
SDS	sodium dodecyl sulphate

SEM	standard error of the mean
T	testosterone
TEMED	tetramethylethylenediamine
TGFα	transforming growth factor alpha
TH1	T-helper 1
TH2	T-helper 2
TNFα	tumour necrosis factor alpha
uNK (cell)	uterine natural killer
VEGF	vascular endothelial growth factor

Chapter 1:

General Introduction and Literature Review

1.1 The Female Reproductive Tract

The female reproductive tract consists of the cervix, uterus, ovaries, fallopian tubes and vagina. This system is the site of the events that occur in the reproductive cycle. The ovaries are the site of ovulation, and are also responsible for much of the steroid action that occurs in the reproductive system. After ovulation, the ovum travels along the fallopian tube to the uterus, and if successfully fertilised, implants in the endometrium, the lining of the uterus. A schematic diagram showing the anatomy of the female reproductive tract is shown in Figure 1.1.

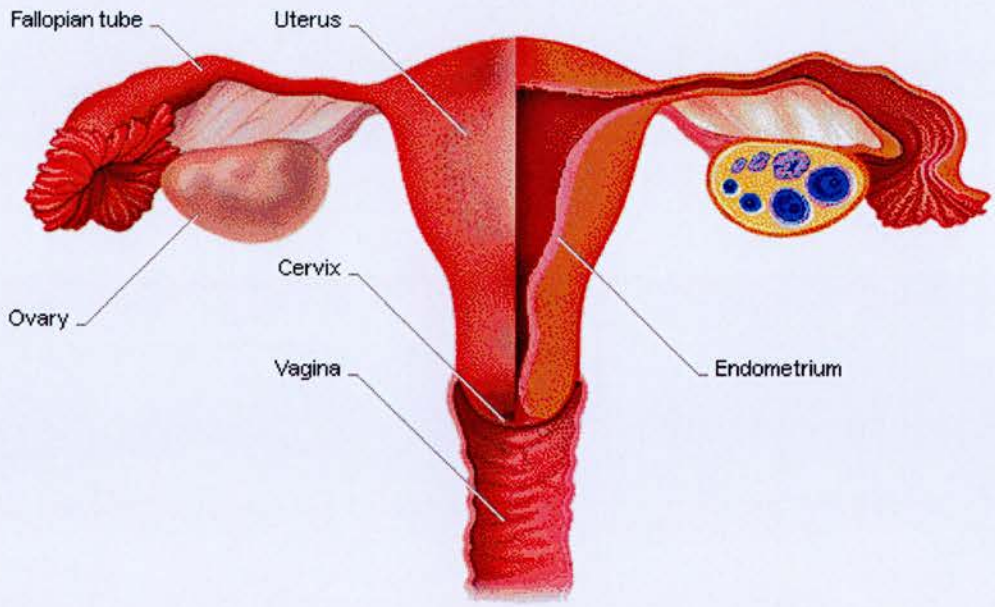


Figure 1.1 Schematic of the female reproductive tract (from Encarta.msn.com)

1.1.1 Regulation of female reproductive tract function

Key events in the female reproductive system, including ovulation, implantation and menstruation are tightly regulated by a number of factors. Two of these factors are the gonadotrophins, luteinising hormone (LH) and follicle stimulating hormone (FSH), produced by the pituitary gland (Heffner, 2006).

A primary regulator of FSH and LH secretion is Gonadotrophin Releasing Hormone (GnRH), released by the hypothalamus. GnRH is released in pulses to stimulate FSH and LH secretion. The frequency of this pulsing controls which hormone is secreted (Heffner, 2006). These hormones are responsible for the control of ovulation, and GnRH is viewed as probably the most important single factor in mediating the events of the reproductive system. Abnormalities in GnRH synthesis or release result in varying degrees of failure of gonadal function (Johnson & Everitt, 2000).

In addition to the gonadotrophins, sex steroid hormones play a vital role in regulation of the menstrual cycle. These are generated by the ovary in females, in response to LH and FSH. The adrenal gland is also a source of sex steroid production. Cholesterol is the precursor of the various steroid hormones. A number of steroid biosynthetic steps are involved in producing the three classes of sex steroids – progestins, estrogens and androgens. LH and FSH act to control the synthesis and release of sex steroids estradiol and progesterone from the ovary, that in turn operate a feedback loop to regulate FSH and LH via GnRH.

The early part of the menstrual cycle, the menstrual and proliferative phases, are characterised by increased estradiol secretion from the ovaries, leading to a peak of estradiol around day 14, triggering a peak of LH and subsequently ovulation (Jones & Lopez, 2006). Following ovulation, a smaller peak of FSH occurs and progesterone levels rise in the secretory phase, peaking around day 21 in the mid secretory stage of the cycle. Control of the menstrual cycle involves intricate feedback loops. GnRH induces secretion of LH and FSH, and estradiol has a negative feedback effect on GnRH secretion. High estradiol levels at the end of the proliferative phase exert positive feedback on GnRH secretion, which in turn induces the LH surge (Jones & Lopez, 2006). In the secretory phase, estradiol and progesterone both cause negative feedback of both FSH and LH secretion. The ratio of estradiol:progesterone is key in determining whether the feedback is negative or positive; a ratio in favour of high estradiol and low progesterone causes positive feedback, whereas low estradiol and high progesterone cause negative feedback (Jones & Lopez, 2006). Thus, the reproductive cycle can be said to be under the control of the hypothalamic-pituitary-gonadal (HPG) axis, whereby the hypothalamus secretes GnRH, which activates release of gonadotrophins from the pituitary. Gonadotrophins act in turn to modulate release of sex steroids which feed back to control GnRH pulsatility. Expression of the gonadotrophins, estradiol and progesterone across the menstrual cycle is shown in Figure 1.2.

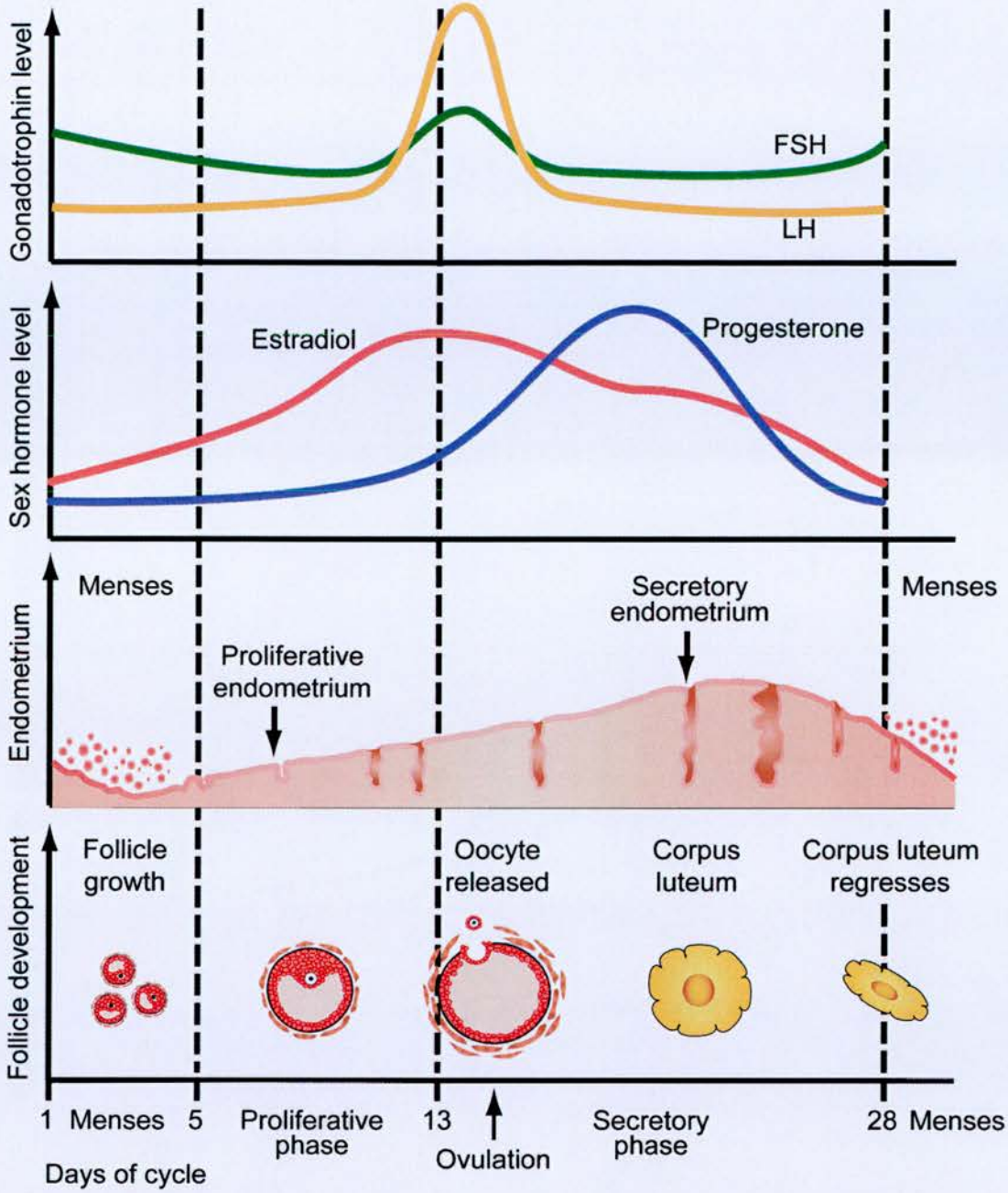


Figure 1.2 Schematic representation of the menstrual cycle, compiled by Ted Pinner, MRC Human Reproductive Sciences Unit, Edinburgh. Stages of the cycle are separated by dotted lines.

The role of androgens in the female reproductive tract is less well understood, however testosterone expression does not vary significantly across the menstrual cycle (Mertens et al., 2001). It has, however, been reported that testosterone can be found in the endometrium at concentrations 10-fold greater than estrogen

(Goebelsmann et al., 1974). Figure 1.3 shows the chemical structures of estrogen, progesterone, and testosterone.

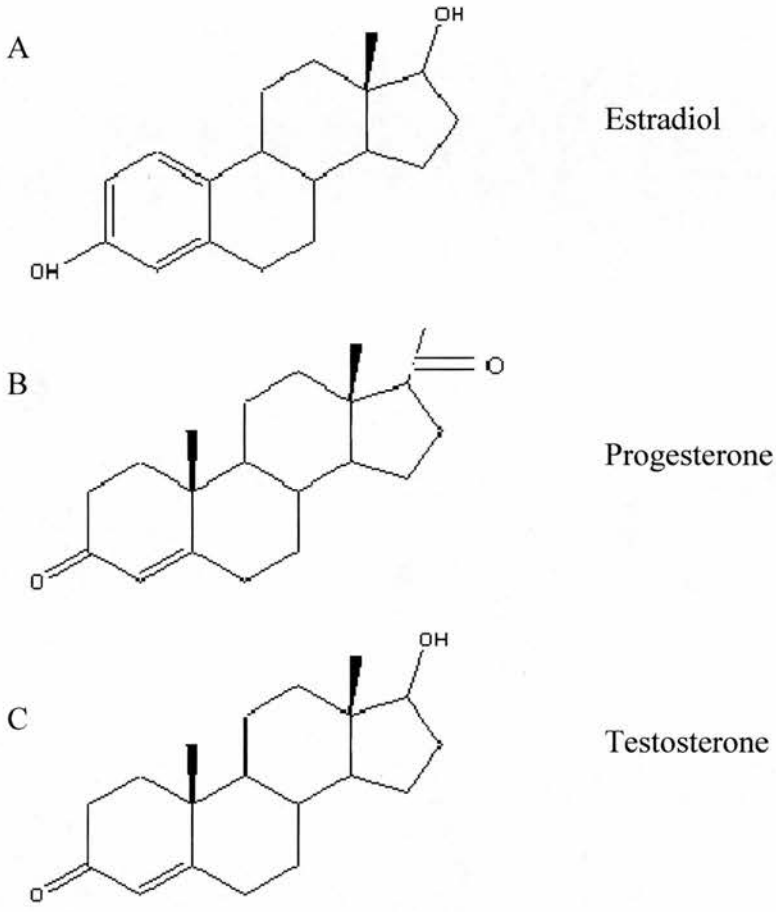


Figure 1.3 Chemical structures of sex steroids. A – Estradiol, B – Progesterone, C – Testosterone

1.1.2 The endometrium

The human endometrium is the lining of the uterus, and the site of embryo implantation. It consists of two layers, namely the basal and functional layers. There are morphological differences between these layers, and it is the uppermost part of the functional layer that is sloughed off and shed each month at menstruation in the absence of pregnancy. Figure 1.4 shows an H&E stained endometrial section

illustrating the functional and basal layers during the early secretory stage of the cycle.

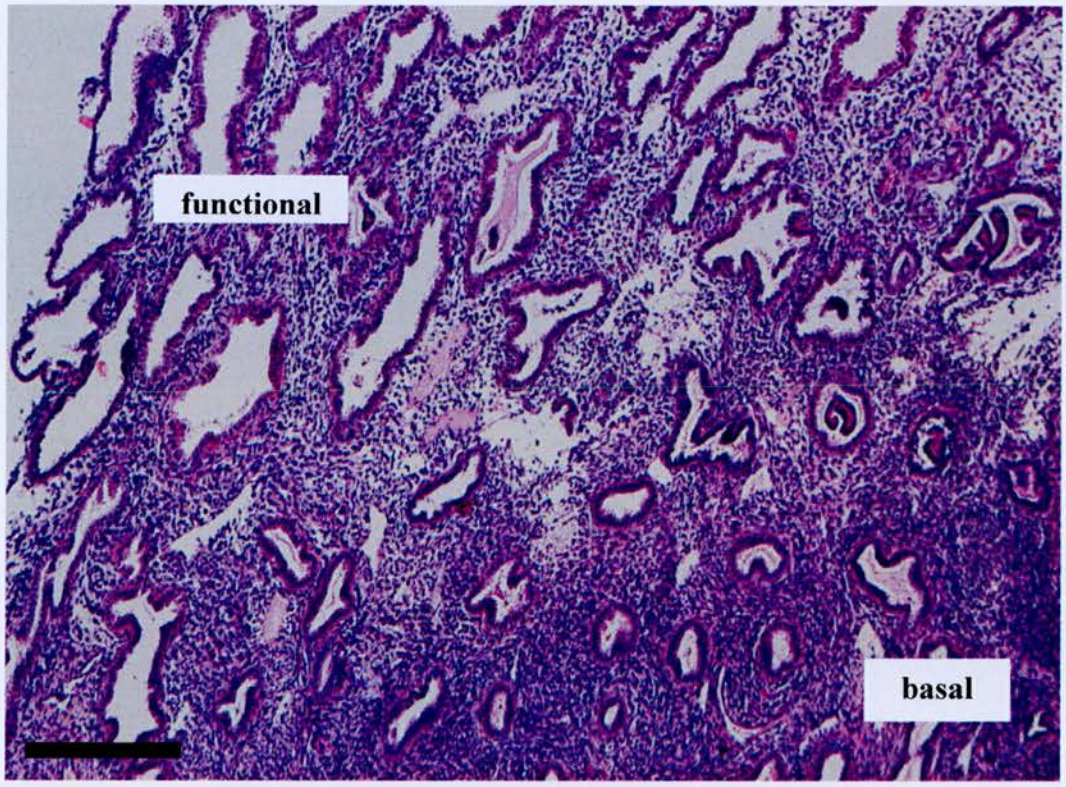


Figure 1.4 H&E stained early secretory stage endometrial section showing functional and basal layers of human endometrium. Scale bar=10 microns.

1.1.2.1 Component cells of the endometrium

The endometrium is a multi-cellular heterogeneous tissue. The major cell components are surface and glandular epithelia (the site of protein secretion), a stromal component consisting of fibroblast-like cells and immune cells, and a well defined vascular system. The vasculature is composed of endothelial cells and smooth muscle perivascular cells. Of particular interest are the spiral arterioles, a vessel type unique to menstruating primates and humans, and thought to have an important role in menstruation. Figure 1.5 shows a schematic diagram showing the

different cell types in the endometrium, and Figure 1.6 shows an H&E stained endometrial section.

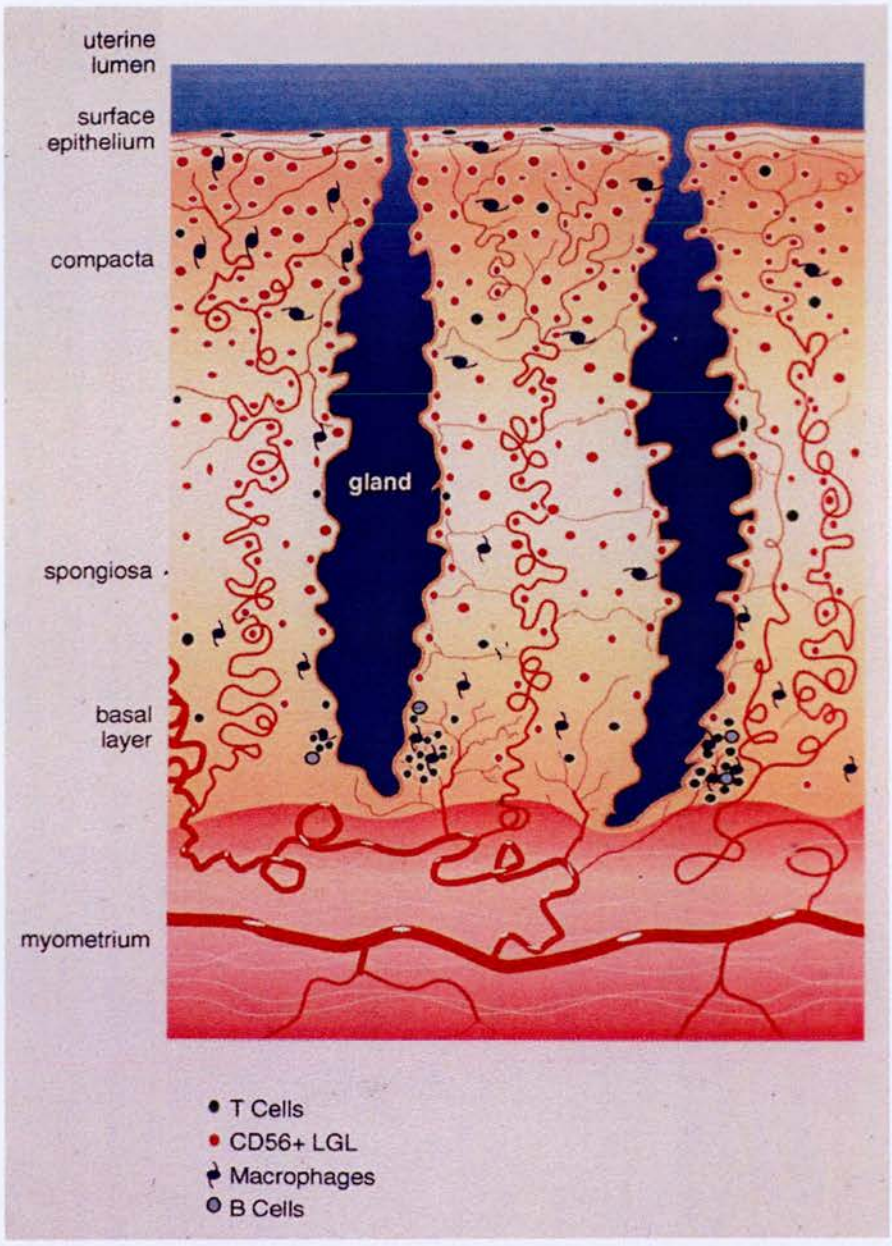


Figure 1.5 Schematic diagram showing endometrial cells. CD56=marker for uNK cells aka large granular lymphocytes (LGL). From Loke & King, 1995.

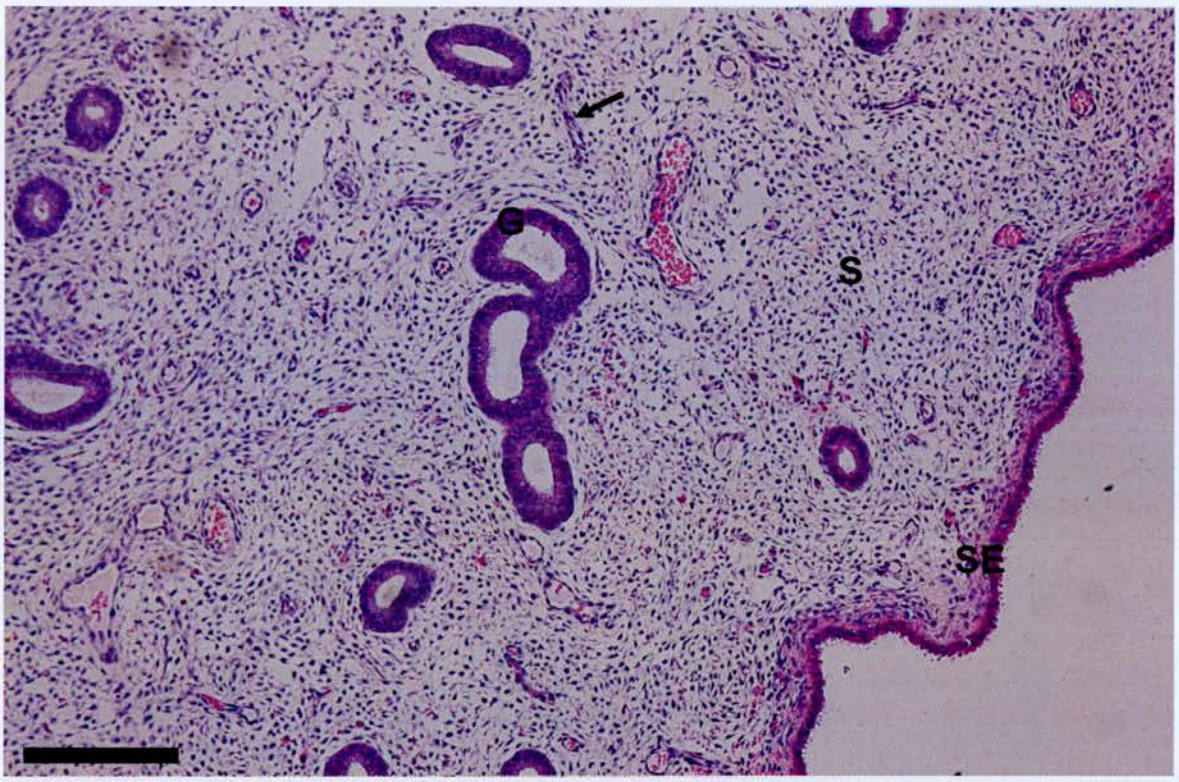


Figure 1.6 H&E stained endometrium showing component endometrial cell types during the proliferative stage of the cycle. G= gland, S=stroma, SE=surface epithelium. Arrow indicates blood vessels. Scale bar=10 microns.

1.1.2.2 Changes In Endometrial Morphology Across The Menstrual Cycle

The endometrium is a dynamic organ that undergoes a number of morphological changes throughout the menstrual cycle (Noyes et al., 1950; Buckley & Fox, 1989).

The first day of menstruation is taken to be day 1 of the cycle, with menses lasting until day 5-7. The first half of the menstrual cycle is known as the proliferative phase, as it is the time when the functional layer regenerates after menstruation. This corresponds to the follicular phase of the ovarian cycle. During this phase, the glands

are small, round, and regular in shape and the stroma is tightly packed in appearance. Ovulation occurs around day 14, following a peak in estrogen secretion.

The subsequent part of the cycle is known as the secretory phase and corresponds to the luteal phase of the ovarian cycle. This phase can be sub-divided into early, mid, and late secretory phases. The glands elongate and become "jagged" in appearance, and the stromal cells become further apart. Progesterone secretion peaks in the mid-secretory phase, signalling to the endometrium to prepare for pregnancy. In the absence of implantation, with luteal regression, progesterone levels fall again and menstruation occurs.

Figure 1.7 shows H&E stained endometrial sections across the menstrual cycle.

If implantation does occur, progesterone levels remain elevated, and a number of morphological changes occur in the endometrium. Stromal cells become enlarged and squamous, and glands become atrophic and less well defined. The epithelial cells lose their cuboidal shape and become flatter. This process is known as decidualisation and pregnant (implanted) endometrium is referred to as decidua. Figure 1.8 shows an H&E stained decidual section.

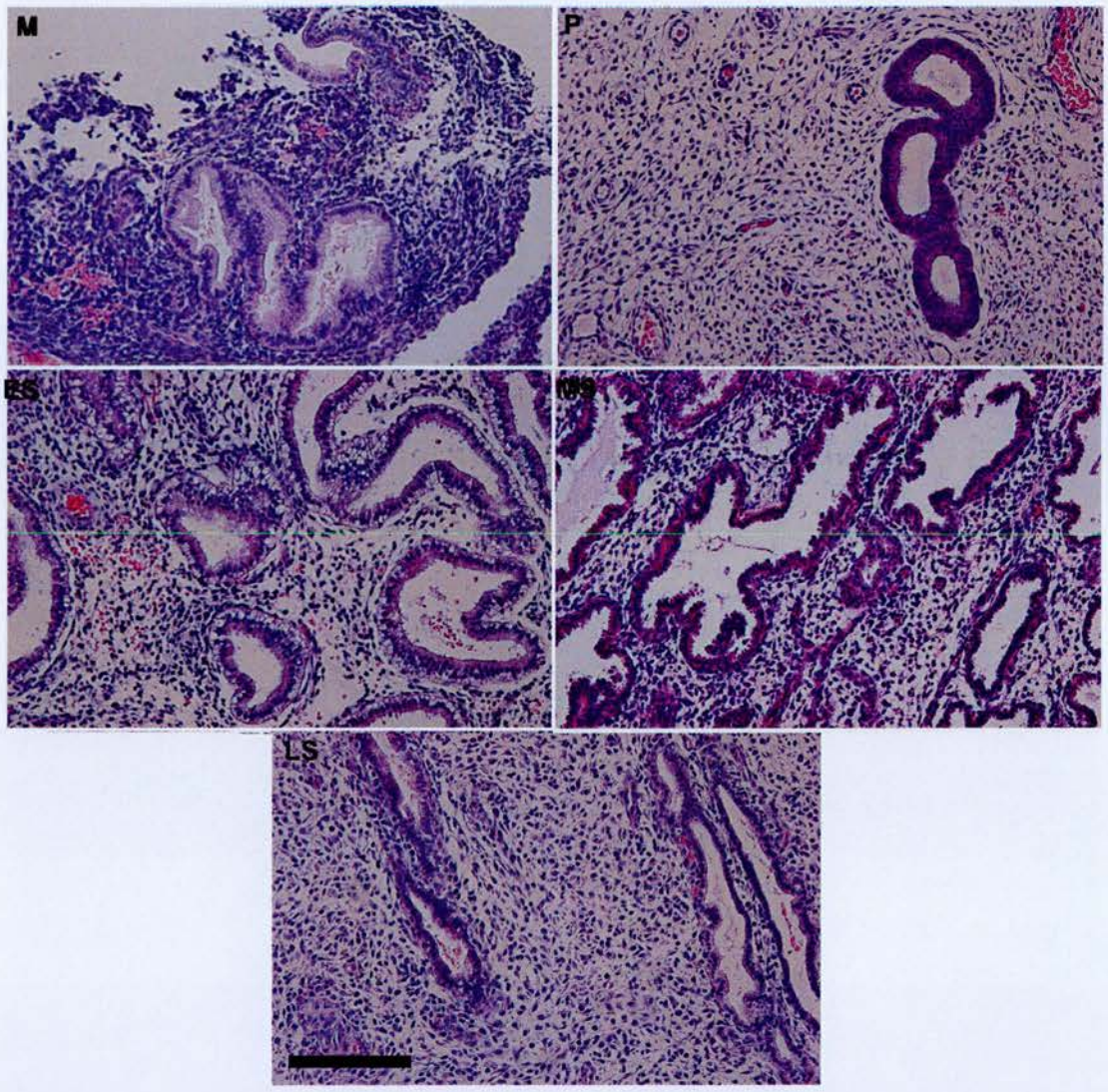


Figure 1.7 H&E stained sections across the menstrual cycle. M=menstrual, P=proliferative, ES=early secretory, MS=mid secretory, LS=late secretory. Scale bar=10 microns.

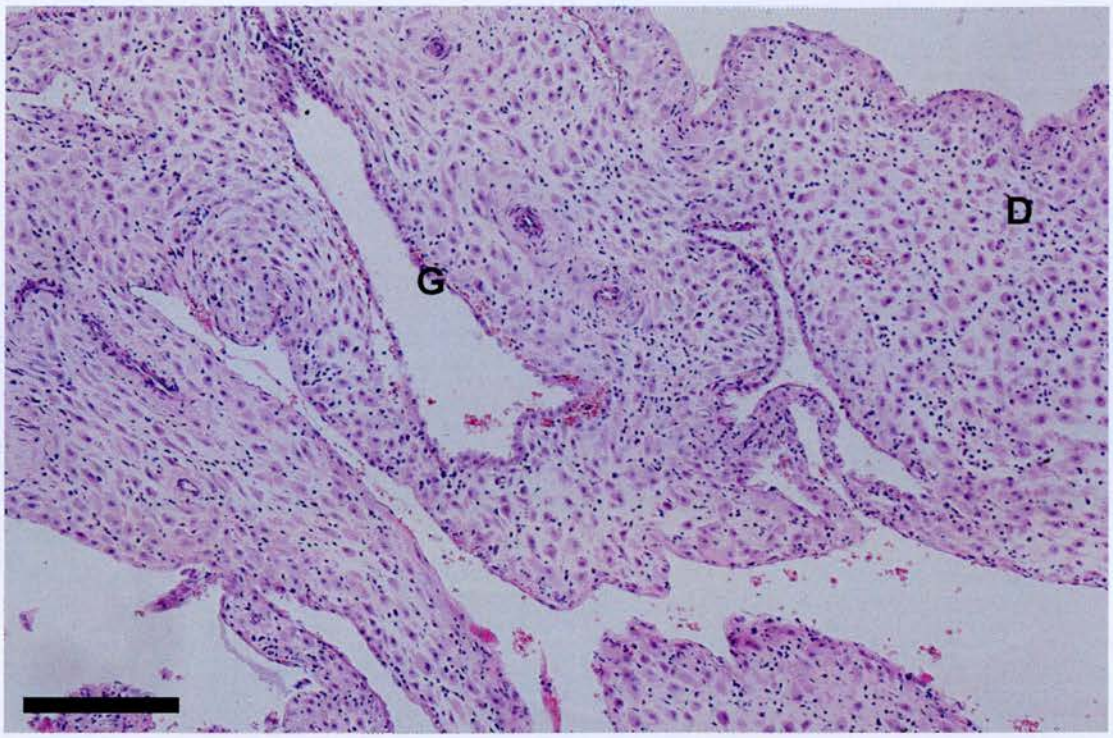


Figure 1.8 H&E Stained decidua section. G=atrophic gland, D=decidualised stromal cells. Scale bar=10 microns

1.1.3 Key Reproductive Events in the Endometrium

The two primary events of importance in the human endometrium are the alternative end-points to each menstrual cycle; implantation or menstruation.

If a successfully fertilised embryo is detected, the endometrium undergoes decidualisation to prepare for implantation. Progesterone levels thus remain high and a degree of tissue remodelling occurs. If fertilisation does not take place, the corpus luteum degrades, progesterone levels decline and menstruation occurs – a process involving tissue injury and repair.

Menstruation can be described as the shedding of the functional layer of the endometrium, accompanied by bleeding. This is induced by steroid hormone withdrawal at the end of each menstrual cycle (Salamonsen, 2003). The basal lamina has been observed to degenerate in the late secretory phase (Roberts et al., 1992) and lesions can be seen on the epithelium at the end of the cycle (Ludwig & Spornitz, 1991). The functional layer is destroyed rapidly leaving blood vessels and glands exposed, thus loss of integrity of vasculature is a consequence of menstruation (Salamonsen, 2003). The endometrium must then be regenerated to prepare to receive an embryo during the next menstrual cycle.

Finn (1986) first proposed that menstruation was an inflammatory process, due to similarities with inflammatory events in other tissues. It is now accepted that this is the case and thought that, due to the remodelling involved, inflammation is also involved in implantation.

The characteristics of inflammation are tissue oedema, the recruitment of inflammatory cell types, and release of pro-inflammatory cytokines (Salamonsen, 2003). The inflammation response that occurs following tissue damage consists of a series of events leading to either a specific immune response or clearance of invading cells (Goldsby et al., 2003). In the case of menstruation, this principle can be applied to the clearing of the shed functional layer of the endometrium. There are three major events that occur in inflammation. Firstly vasodilation, where the diameter of capillaries increases. Capillary permeability also increases leading to an influx of

fluid and immune cells into the tissue. This then leads to oedema. There is then an influx of phagocytes, and release of pro-inflammatory cytokines (Salamonsen, 2003).

Inflammation is mediated by a complex series of events, not all of which are well understood (Goldsby et al., 2003). When the inflammatory response subsides, the process of tissue repair and regeneration begin. In most cases, this involves the formation of scar tissue however the endometrium does not display this characteristic.

It has been shown that there is an increase in leukocyte population prior to menstruation (Salamonsen & Woolley, 1999). Leukocytes make up around 40% of the total cells in the endometrial stroma (Salamonsen & Woolley, 1999). Specifically, there are large numbers of neutrophils, eosinophils, macrophages and monocytes. This is thought to be due to migration of cells from the blood, and cell proliferation within the tissue. Leukocyte phenotype and state of activation play a role in determining endometrial function (Salamonsen et al., 2002). This influx of leukocytes occurs when progesterone is up-regulated, suggesting this event is progesterone-dominated. However, most leukocytes lack progesterone receptors (King et al., 1996; Monsour et al., 1994; Marx et al., 1999; Tabibzadeh & Satyaswaroop, 1989), thus cells which do express PR, the stromal, epithelial and endothelial cells mediate the leukocyte influx. Salamonsen et al. (2002) proposed that these leukocytes may act as effector cells to mediate destruction and remodelling of endometrial tissue along with factors produced by endometrial cells.

Glucocorticoids are steroid hormones that play an important role in the inflammatory process. The primary active glucocorticoid in humans is cortisol, which has an anti-inflammatory effect. Cortisol is secreted by the adrenal gland and travels to the site of action via the circulatory system. Local modulation of cortisol expression also occurs by a number of factors which will be discussed in section 1.2.

Cortisol acts by binding to its receptor and activating downstream signalling pathways. Progesterone also has some anti-inflammatory activity (van der Burg & van der Saag, 1996). As progesterone has a relatively weak anti-inflammatory action, increased local cortisol levels are required to modulate the inflammatory response in the ovary (Hillier & Tetsuka, 1998). As there are many similarities between the ovary and endometrium, i.e. they both go through inflammation-associated cycles of tissue injury and repair, the expression and action of cortisol in the endometrium is an area of interest.

1.1.4 Endometrial Leukocytes

Leukocytes make up around 40% of the total cells in the endometrial stroma (Salamonsen & Woolley, 1999). Specifically, there are large numbers of neutrophils, eosinophils, macrophages and monocytes. This is thought to be due to migration of cells from the blood, and cell proliferation within the tissue. Leukocyte phenotype and state of activation play a role in determining endometrial function (Salamonsen et al., 2002). Salamonsen et al. (2002) also proposed that these leukocytes may act as

effector cells to mediate destruction and remodelling of endometrial tissue along wih factors produced by endometrial cells.

The number and type of endometrial leukocytes vary across the menstrual cycle. The main types of leukocytes found in the endometrium are T cells, B cells, mast cells, macrophages and neutrophils (Jabbour et al., 2006). There is also a population of phenotypically unique lymphocytes with a CD56+, CD16-, CD3- phenotype. These are uterine Natural Killer (uNK) cells, and their numbers increase in the late secretory phase of the cycle, and again in first trimester decidua (King et al., 1989). It is unknown whether this increase in uNK cell expression is due to *in situ* proliferation or migration from a circulatory population. A possible precursor cell type has been identified by Lanier et al. (1986); in the form of a subset of peripheral NK cells that express a similar phenotype to uNK cells. However Kammerer et al. (1999) found CD56+ uNK cells to express the proliferation marker Ki67, suggesting these cells undergo proliferation.

There are few neutrophils in the endometrium, but numbers do increase pre-menstrually (Poropatich et al., 1987). It is thought that progesterone withdrawal may be the key to neutrophil influx. Leukocytes are responsible for the synthesis of immunoregulatory cytokines that initiate immune responses (Staples et al., 1983; King et al., 2003). Macrophages increase in number in the late secretory phase, and are thought to be under indirect control by sex steroids. They could possibly have a role via matrix metalloproteinases in endometrial breakdown and menstruation (Jabbour et al., 2006).

1.2 Steroid hormone metabolism and pre-receptor signalling

The functional and morphological changes that the human endometrium undergoes in each menstrual cycle to prepare for pregnancy are the result of a complex series of molecular events, not all of which are well defined. The events are under the control of sex steroids and their cognate receptors. In addition to control by sex steroids, glucocorticoids are also involved in key reproductive events.

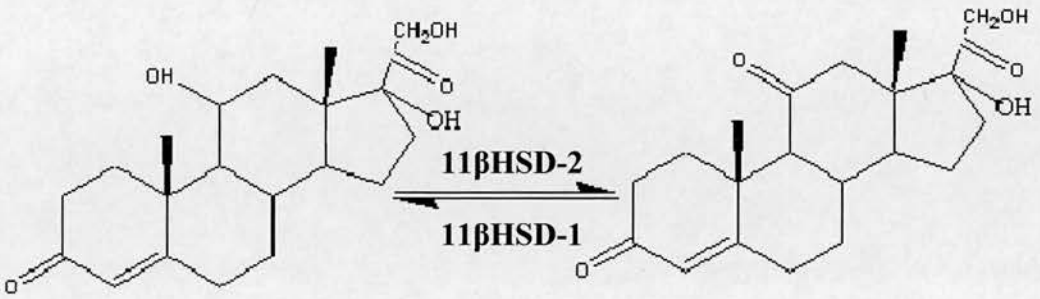
The local availability of steroid hormones is regulated by a number of factors, primarily locally expressed steroid metabolising enzymes such as the hydroxysteroid dehydrogenases (HSDs). The majority of HSDs act in an intracrine manner to regulate local steroid expression (Labrie., 1991). In the context of this thesis, of particular interest in the endometrium are the enzymes that control glucocorticoid metabolism, the 11β-hydroxysteroid dehydrogenases (11βHSDs), and those that control sex steroid metabolism, the 3α-hydroxysteroid dehydrogenases (3αHSDs), 3β-hydroxysteroid dehydrogenases (3βHSDs) and 17β-hydroxysteroid dehydrogenases (17βHSDs).

1.2.1 Glucocorticoid Metabolism

1.2.1.1 11βHSDs

The 11β-hydroxysteroid dehydrogenase (11βHSD) family are enzymes that catalyze glucocorticoid metabolism, regulating the availability of active glucocorticoid

(cortisol in humans, corticosterone in rodents) and their inactive metabolites (cortisone and dehydrocorticosterone, respectively). The chemical structures of cortisol and cortisone are shown in Figure 1.9.



Cortisol Cortisone

Figure 1.9 Reactions catalysed by 11βHSD-1 and -2.

Glucocorticoids are crucial to the inflammatory response that accompanies tissue remodelling. Pro-inflammatory cytokines elicit an increased resynthesis of cortisol from cortisone in many tissues, suggesting local cortisol activation, by 11βHSDs. This was first shown by Escher et al. (1997) in renal glomerular mesangial cells. Studies on up- and down-regulation of cortisol levels by 11βHSD-1 and -2 in inflammatory responses at ovulation (Hillier & Tetsuka, 1998), in kidney, liver and fat (Seckl & Walker, 2004), and in bone (Cooper et al., 2001) have been described. Deficiencies in the regulation of 11βHSD can cause problems including liver failure, obesity, metabolic and cardiac disorders. In the reproductive tract, tissue injury and repair occur on a cyclical basis, and conditions including ovarian cancer and heavy and painful menstrual bleeding are associated with this. Rapid resolution of inflammation at these times of tissue injury and repair is thus essential, and this is likely to be mediated by cortisol, which in turn is mediated by the 11βHSDs.

There are two characterized 11βHSDs in humans, encoded by two separate genes, located on different chromosomes, and with only 14% homology (Albiston et al., 1994). 11βHSD-1 was first cloned in 1991 by Tannin et al. This gene was found to be expressed at greatest levels in the liver and acts predominantly as an NADPH-dependent reductase, although in the presence of $NADP^+$ it can also act as a dehydrogenase converting cortisol to cortisone. Human 11βHSD-2 was cloned in 1994 by Albiston and colleagues (Albiston et al., 1994) and was first identified in kidney. This enzyme is a unidirectional NAD^+-dependent dehydrogenase.

1.2.1.1 11βHSD expression in the endometrium

Studies have been carried out on the expression patterns of 11βHSD enzymes in the female reproductive tract. Studies on the expression of 11βHSDs in the endometrium were conducted by Smith et al. (1997). They performed immunohistochemistry on endometrial tissue samples, collected at curettage from women with regular menstrual cycles, supported by western immunoblotting and activity assays to build an expression profile. It was found that 11βHSD-2 protein was expressed in the luminal and glandular epithelia, with heterogeneous expression in some tissue sections. Levels of 11βHSD-2 activity were found to be higher in the secretory phase than in proliferative phase endometrium. The activity of 11βHSD-1 in endometrium was also assayed with constant levels of activity found across the cycle; however, the overall level of 11βHSD-1 activity was lower than that of 11βHSD-2. This study did not investigate 11βHSD-1 protein across the menstrual cycle, nor were any mRNA studies included.

Hitherto there has been no definitive study of the expression profile of 11βHSD-1 protein in endometrium, due to the lack of an antibody suitable for western immunoblotting and immunohistochemistry of endometrial samples. Arcuri and colleagues (Arcuri et al., 1996) studied the effects of various steroid treatments on endometrial stromal cells and found progesterone increased expression of 11βHSD-1. Estrogen alone had no effect while the combination of estrogen and progesterone potentiated the effect of progesterone. They also found 11βHSD-1 up-regulation to be a feature of decidualisation in these cultured cells. Koyama and Krozowski (2001) assayed the enzymic activity of Ishikawa cells, an endometrial carcinoma cell line. They found 11βHSD activity increased in a dose-dependent manner when measured with cortisol as substrate. NAD^+ was preferred to $NADP^+$ as a cofactor, suggesting predominant 11βHSD-2 activity.

1.2.2 Regulation of Glucocorticoid Signalling

The steroid metabolising enzymes are tightly controlled by a number of factors, including steroid hormones themselves, cytokines, chemokines, and many other factors. Of particular interest in the context of this thesis is the regulation of the 11βHSD system and glucocorticoid signalling by cytokines.

1.2.2.1 Cytokines in the Endometrium

It has now been accepted following the work of Finn (1986) that implantation and menstruation are inflammatory events. Cytokines are among the factors involved in

these events (Kelly et al., 2001). Cytokines are produced by many cell types, including epithelia and endothelia (Tabibzadeh, 1994), and have been described to be protein cell regulators involved in physiological responses. There are many cytokine factors, including interleukins, colony-stimulating factors and tumour necrosis factors. Cytokines are able to act in intracrine, autocrine and paracrine manners (Tabibzadeh, 1994; Labrie et al., 1988; Labrie, 1991). It was Labrie et al., (1988), who first coined the term intracrinology to describe the synthesis of active steroids in peripheral target tissues where the action is exerted in the same cells where synthesis takes place without release of the active steroids in the extracellular space and general circulation.

A number of cytokines have been reported to have involvement in endometrial processes via the hypothalamic-pituitary-gonadal (HPG) axis, including interferon-γ, interleukin-1 (IL-1), IL-6, tumour necrosis factor-α, transforming growth factor-β and others (reviewed by Tabibzadeh, 1994). Tabibzadeh & Sun (1992) investigated expression of a number of cytokines in endometrium across the menstrual cycle. They found IL-1α protein to be expressed throughout the cycle, in both the glands and stroma. IL-1β was also detected, but at much lower levels. Transforming growth factor-α (TGFα) was expressed in the epithelial, stromal and endothelial cells; IL-6 was strongly expressed in the epithelia and weakly expressed in stromal cells. Levels of IL-1 in human serum were found to be greatest in the secretory phase (Cannon & Dinarello, 1985).

1.2.2.2 Interleukin-1

There are two isoforms of IL-1, IL-1α and IL-1β. Both isoforms act via the IL-1R receptor (IL-1R), of which there are two known types, designated type 1 and type 2. Both IL-1 isoforms bind IL-1R type 1, whereas type 2 binds IL-1β (Corlotta et al., 1993). IL-1 has been implicated in the key inflammatory events in the endometrium. IL-1 has been postulated to have a role in blastocyst implantation and can be identified in both trophoblast and decidual stromal cells (Simon et al., 1994; 1995).

There has also been a suggestion that IL-1α stimulates MMP production and release, thereby having an effect on the initiation of menstruation (Cole et al., 1995; Rossi et al., 2005). Further indications of the involvement of IL-1 in the endometrium can be inferred from reports of alterations in IL-1 production in conditions affecting the endometrium and decidua such as pre-eclampsia. Pre-eclampsia is a likely consequence of impaired trophoblast invasion. This condition can lead to maternal and fetal death. Endometriosis, a common endometrial disease whereby endometrial lesions form ectopically, has also been linked to altered IL-1 production (Mori et al., 1992; Lockwood et al., 2006). This condition can cause severe pain and is associated with fertility problems.

Previous studies have focussed on the effect of IL-1α on the female reproductive tract, in particular in the ovarian surface epithelium (OSE). Yong et al. (2002) found 11βHSD-1 expression to be increased in the presence of IL-1α in cultured OSE cells. This was followed up by the work of Rae et al. (2004a), who found 11βHSD-1 to be

up-regulated following IL1α treatment, but found the same treatment to have no effect on 11βHSD-2 expression. Il-1α caused a small but significant increase in GR expression. A further interesting finding of this study was that the addition of cortisol in the presence of IL-1α caused a further enhancement of 11βHSD-1 expression (Rae et al., 2004a). However little is currently known about the effect of IL-1α on glucocorticoid metabolism and associated signalling in the endometrium.

1.2.3 Sex Steroid Metabolism

A number of enzymes are involved in the metabolism and control of sex steroids. Of particular interest in this thesis are the 3βHSDs, which convert pregnenolone to progesterone and are involved in androgen metabolism, 3αHSDs (AKRs) and 17βHSDs, which have many roles in the metabolism of androgens and estrogens.

1.2.3.1 3βHSDs

3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3βHSD) catalyses the conversion of Δ^4-3-ketosteroids from Δ^5-3β-hydroxysteroids e.g. pregnenolone and dehydroepiandrosterone (DHEA). This is a key step in synthesis of androgens, estrogens, mineralocorticoids and glucocorticoids (Readhead et al., 1983). Additionally, 3βHSDs can also act on downstream sex steroids, predominantly androgens, converting dihydroepiandrosterone (DHEA) to androstenedione, androst-5-ene-3β,17β-diol to testosterone, and dihydrotestosterone (DHT) to 3β-diol. 3βHSD is also capable of acting on 17α-hydroxypregnenolone, converting it to the more

active 17α-hydoxyprogesterone. These reactions are summarised in the diagram in Figure 1.10.

There are two known human isoforms of 3βHSDs, namely type 1 and type 2. Human type 1 was first cloned from placenta by Thomas et al., (1989), while human type 2 3βHSD was identified in adrenal glands and gonads of humans. Two functional human 3βHSD genes have been identified, part of a closely related gene family (Rheaume et al., 1991). The 3βHSD isoforms are part of the short-chain dehydrogenase/reductase (SDR) gene superfamily, the same family as the 11βHSDs. There have been three reports of 3βHSD expression in the human endometrium. Rhee et al. (2003) recently published a report whereby they found 3βHSD expression in the glandular epithelium of the endometrium, with the intensity of immunoreactivity increasing across the cycle. Staining was weak in proliferative phase endometrium, moderate in secretory phase, and intense in decidua; the expression of 3βHSD increased as progesterone levels increased. This was investigated by immunohistochemistry and confirmed by RT-PCR.

Seki et al., (1987), cited by Rhee et al. (2003), and Tang et al. (1993) studied enzyme activity of 3βHSD in human endometrium. Tang et al. (1993) found that 3βHSD was indeed active in human endometrium, but did not examine expression across the menstrual cycle.

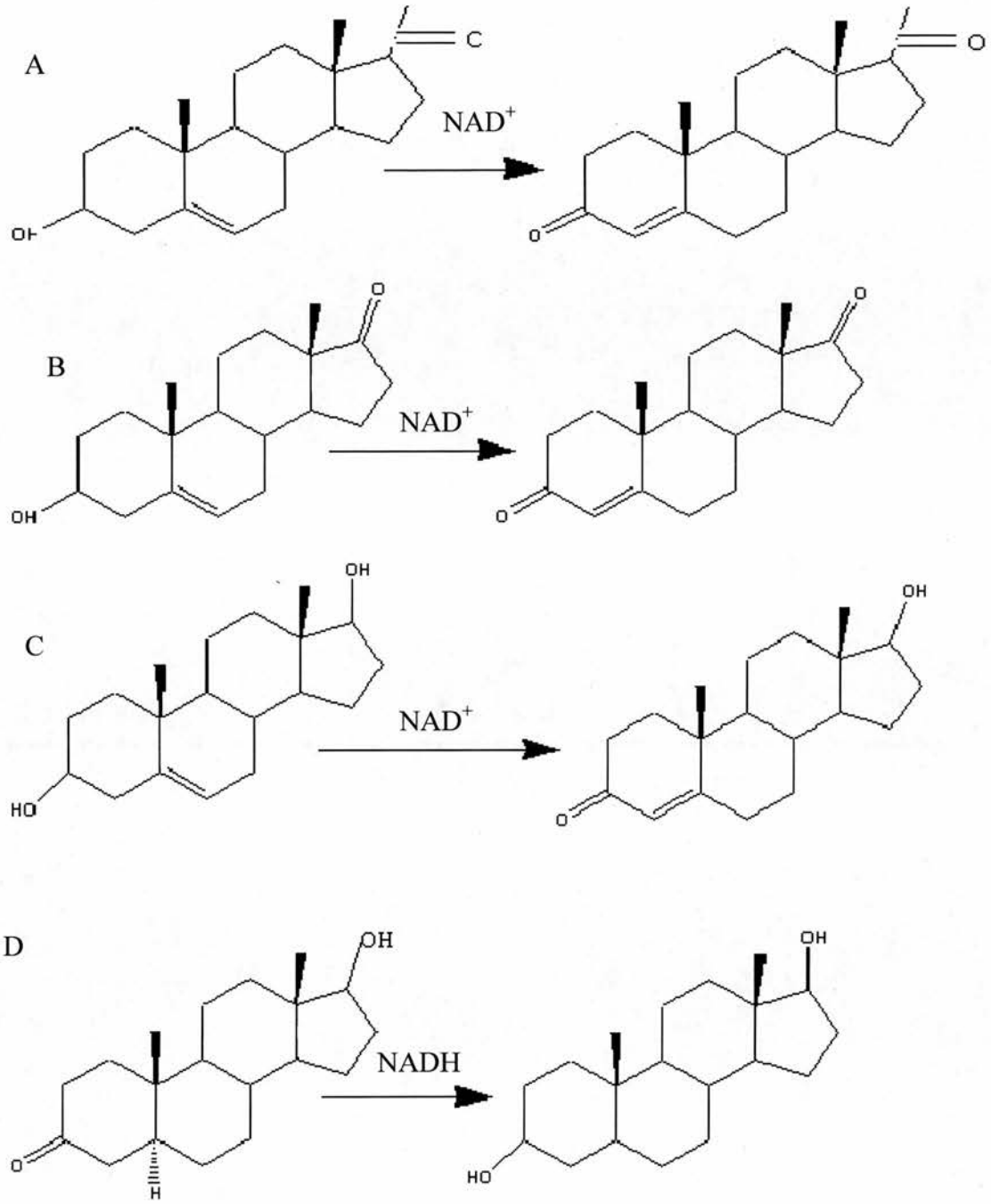


Figure 1.10 Reactions catalysed by 3βHSD. A – pregnenolone to progesterone, B - DHEA to androstenedione, C – androstenediol to testosterone, D – DHT to 5α-androstanediol

1.2.3.2 Aldo-keto Reductases

The aldo-keto reductase enzyme superfamily are closely related to the SDR family. The AKR1C family (3αHSDs) are involved in many steroid metabolising reactions. They are a promiscuous group of enzymes; their ability to act on a particular substrate is governed by the relative availability of potential substrates and the appropriate regulatory cofactors. Among the many substrates 3αHSD/AKR1C enzymes can act on, are the three main groups of sex steroids, androgens, estrogens and progestins, which are all of relevance in the endometrium.

There are four identified members of the AKR1C family, designated AKR1C1-1C4. Each AKR1C enzyme has a number of alternative names, as well as being named 3αHSDs; AKR1C1 is also known as 20αHSD, and AKR1C3 is 17βHSD-5. Table 1.1 depicts the alternative nomenclature for the AKR1C enzymes.

Table 1.1: AKR1C nomenclature

AKR1C enzyme	Alternative names
AKR1C1	20α(3α)HSD DD1
AKR1C2	3αHSD-3 Bile acid binding protein DD2
AKR1C3	3αHSD-2 **17βHSD-5** DDx Prostaglandin F_2 synthase
AKR1C4	3αHSD-1 chlordecone reductase DD4

From now on the AKR1C nomenclature will be used for these enzymes.

Table 1.2 depicts the many reactions performed by the AKR1C enzymes, and highlights those that predominate in intact cells.

Table 1.2: Reactions of AKR1C enzymes.

Enzyme	Substrate	Product
AKR1C1	Progesterone 5α-dihydrotestosterone	20α-hydroxyprogesterone Androstane-3β, 17β-diol
AKR1C2	5α-dihydrotestosterone Androstane-3β, 17β-diol	Androstane-3, 17β-diol 5α-dihydrotestosterone
AKR1C3	Androstendione Estrone 5α-dihydrotestosterone Progesterone PGD_2 Androstane-3α, 17β-diol Androstane-3β, 17β-diol Deoxycortisol	Testosterone Estradiol Androstane-3, 17β-diol 20α-hydroxyprogesterone 9α, 11βPGF 5α-dihydrotestosterone Androsterone 20α-dihydrodeoxycortisol
AKR1C4 (in liver)	5α/5β-dihydrosteroids	5α/5β-tetrahydrosteroids

Penning et al. (2000) have found all 4 AKR1C enzymes to be expressed at low levels in the uterus, by RT-PCR. AKR1C1 and AKR1C2 were found to be expressed in the lung, whereas AKR1C3 (17βHSD-5) was highly expressed in the mammary gland, and AKR1C4 only detectable at significant levels in the liver. This correlates with previous studies which have declared AKR1C4 to be almost entirely liver-specific (Penning et al., 2000)

Ito et al. (2006) have reported on the expression of AKR1C3 (17βHSD-5) in human endometrium. They found the protein to be expressed in both proliferative and secretory phases of the endometrium; however there is currently little else known about the expression of the AKR1C enzymes in the human endometrium.

1.2.3.3 17βHSDs

There are 13 human 17βHSD enzymes currently identified. These are a large family of enzymes with a wide variety of steroid metabolising activities. Various 17βHSDs are involved in the regulation of sex steroids. Of particular interest here is 17βHSD-2, which performs the reverse of the reactions catalysed by AKR1C3/17βHSD-5, predominantly the inactivation of estradiol and testosterone.

There have been a number of studies on 17βHSD-2 in the endometrium reported. Casey et al. (1994) and Burton et al. (2003) described 17βHSD-2 protein to be expressed in the glandular epithelia of the endometrium in the secretory stage of the cycle, with negligible expression in proliferative endometrium. However a recent molecular phenotyping study (Talbi et al., 2006) showed 17βHSD-2 to be highly up-regulated in early secretory endometrium compared to the proliferative phase, but down-regulated in mid-secretory endometrium compared to the early secretory stage.

Figure 1.11 shows a summary of the key endometrial steroid metabolism pathways involving the enzymes discussed in this thesis.

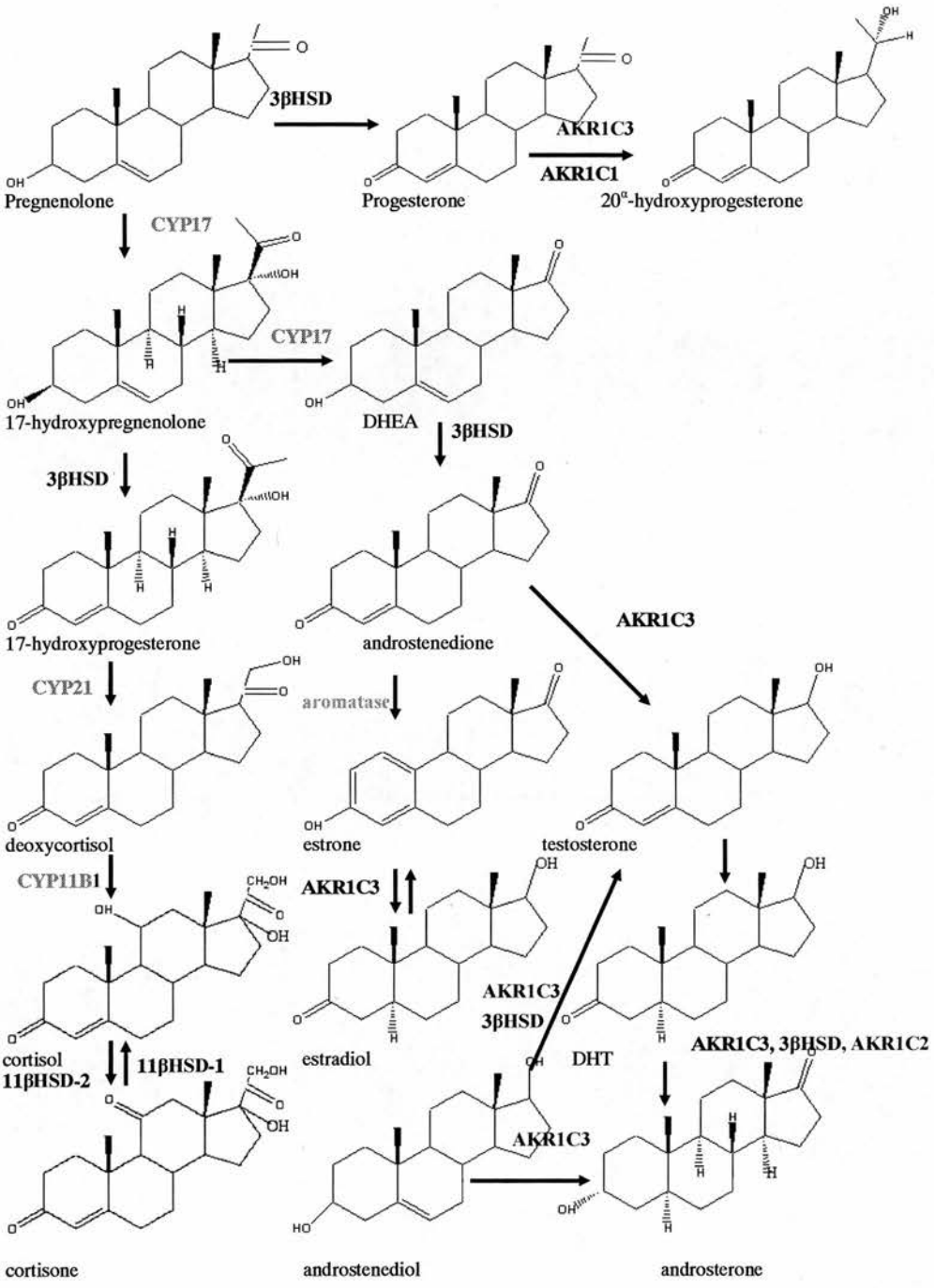


Figure 1.11 Key steroid metabolic pathways in human endometrium. Enzymes of interest in this thesis are shown in bold, with those that have not been investigated in human endometrium in grey.

1.2.4 Steroid hormone receptors

1.2.4.1 Glucocorticoid Receptors

There are separate receptors for glucocorticoids and mineralocorticoids, GR and MR respectively. However cortisol can bind to the MR and thereby act as a mineralocorticoid.

GR is almost ubiquitously expressed in all human tissues and organs (Kino & Chrousos, 2001). There are two alternative splicing variants, GRα and GRβ. GRα is the classical GR, whereas GRβ is thought to act as a dominant negative inhibitor of GRα and does not bind glucocorticoids (Kino & Chrousos, 2001). GRα is expressed in endometrial stromal cells across the menstrual cycle (Bamberger et al., 1995; Henderson et al., 2003). GR is not expressed in the glandular or surface epithelium in endometrium, but has been observed in these cells in first trimester decidua (Henderson et al., 2003).

Cortisol also binds with high affinity to the mineralocorticoid receptor (Stewart & Mason, 1995). The function of MR is to mediate aldosterone effects and thus regulate the balance of sodium and water in the body. In the CNS, MR has been proposed to have a role in regulation of the stress response and feedback control of the hypothalamus-pituitary-adrenal axis (de Kloet et al., 1990). Activation of MR by excess ligands, usually resulting from cortisol binding, can lead to conditions such as apparent mineralocorticoid excess. This can cause persistent hypertension and

hypokalemia, thus 11βHSD-2 is often found to be co-localised with MR (Hirasawa et al., 2000). MR expression in human endometrium or first trimester decidua has not been previously reported.

Cortisol has a high affinity for MR and can act as a potent mineralocortocoid when it binds to MR. Excess binding of cortisol to MR can cause a number of disorders, in particular apparent mineralocorticoid excess and hypertension (Wilson et al., 1995, cited by Smith et al., 1997). In order to prevent this, 11βHSD-2 has been found to be co-localised with MR in a number of tissues including kidney (Edwards et al., 1988), colon (Funder et al., 1988), eye (Suzuki et al., 2001) and placenta (Hirasawa et al., 2000).

1.2.4.2 Sex Steroid Receptors

The receptors for the products of the reactions catalysed by the 3βHSD and AKR1C enzymes are predominantly the androgen receptor (AR), estrogen receptors (ERα and ERβ) and the progesterone receptor (PR). The expression of all these receptors in the endometrium has been well characterised.

There are two PR isoforms, PRA and PRB (Tung et al., 1993). Both are active, with PRB thought to be the more transcriptionally active form (Wen et al., 1994). PRA is thought to act as a repressor of PRB function. Studies have been conducted on these receptor isoforms using knockout mice. PR knockout (PRKO) mice are unable to support implantation (Lydon et al., 1995). Unopposed estrogen action causes

epithelial cells to become hyperplastic (Conneely et al., 2001). Specific PR-A and PR-B knockouts have shown that the two isoforms have distinct uterine functions (Mulac-Jericevic et al., 2000, 2003). PR-A knockout mice show inhibition of endometrial stromal cell decidualisation and it has been proposed that PR-A has a crucial role in the process of decidualisation. PR-B knockout mice display normal antiproliferative and implantation-associated responses to progesterone, thus PR-A is necessary and sufficient to mediate these responses (Mulac-Jericevic et al., 2000). PR-B activation in PR-A knockout mice surprisingly led to abnormal progesterone-dependent induction of epithelial cell proliferation, thus PR-A is required to inhibit potentially adverse effects of PR-B such as this abnormal proliferation. Aberrant ratios of PR-A to PR-B have been associated with endometrial cancers (Arnett-Mansfield et al., 2001; DeVivo et al., 2002).

PR expression in the endometrium is induced by estrogen in the proliferative phase. Both PR isoforms have been identified in the stromal and epithelial cells in this phase. Progesterone secretion increases following ovulation, and causes PR expression to be down-regulated, particularly in the glandular epithelium (Chauchereau et al., 1992). PR has been reported in the stromal, perivascular and endothelial cells in first trimester decidua (Perrot-Applanat et al., 1994; Wang et al., 1992). Wang et al. (1998) studied the PR-A and PR-B isoforms in human endometrium and found both subtypes to be present in glands and stroma in the proliferative phase, and both were dramatically reduced in the glands during the secretory phase. PR-B was in fact not detectable at all in the secretory phase (Wang et al, 1998).

There are two distinct forms of ER, ERα and ERβ, encoded by separate genes. ERα is highly expressed in both the glands and stroma of the endometrium in the proliferative phase of the menstrual cycle (Lessey et al., 1988; Snijders et al., 1992; Garcia et al., 1988). ERα expression is down-regulated in the secretory phase in response to elevated progesterone levels. Both ERα and PR have been reported to be expressed in perivascular cells (Perrot-Applanat, 1988; Critchley et al., 2001a).

Narvekar et al. (2006) have shown PR expression to be reduced in the endometrial surface epithelium following treatment with the antiprogestogen mifipristone. PR expression was greatly increased following mifepristone administration, however the treatment had no effect on ER expression.

ERβ is expressed in the glandular epithelium and stromal cells, and also in endothelial cells, unlike ERα (Critchley et al., 2001a). ERβ has also been shown to be expressed at greatest levels in the proliferative phase and down-regulated in the secretory phase (Taylor & Al-Azzawi, 2000; Critchley et al., 2001a). Two ERβ splice variants have been identified. ERβ1 and ERβcx/β2 are both expressed in the human endometrium, with expression of ERβcx/β2 being less intense than that of the full length variant ERβ1 (Critchley et al., 2002). ERβ1 expression does not differ across the menstrual cycle, however a reduction in ERβcx/β2 levels was observed in the glands of the functional layer of the endometrium in the mid-secretory phase. ERα levels are very low in first trimester decidua (Lessey et al., 1988); however ERβ can be detected in decidual stromal cells (Milne et al., 2005).

AR expression has been studied in human endometrium; however reports of its expression pattern have varied. Horie et al. (1992) and Ito et al. (2002) found AR to be expressed in both glandular and stromal cells, whereas other authors have reported stromal localisation of AR (Maia et al., 2001; Mertens et al., 2001; Slayden et al., 2001; Burton et al, 2003). Slayden et al. (2001) have also reported AR to be expressed in a similar manner in the endometrium to ER and PR, greatest expression in the estrogen-dominated proliferative phase and down-regulated in the secretory phase. AR is expressed in decidual stromal cells and endothelial cells (Milne et al., 2005). The role of androgens and the AR in the endometrium is not yet well understood, however AR has been shown to be upregulated following antiprogestogen treatment (Brenner et al., 2003), and this upregulation is thought to have an antiproliferative effect potentially by suppressing estrogen-dependent growth factors (Brenner et al., 2003).

1.3 Exogenous steroid manipulation of the endometrium

A number of synthetic steroids are commonly used clinically in the management of sub-fertility, contraception and treatment of menstrual disorders. These take a variety of forms, including synthetic progestins and estrogens, and substances that act as agonists or antagonists of endogenous steroids. These steroid treatments often alter the morphology and gene and protein expression profile of the endometrium, however there is still much to learn about these effects.

Of particular interest in this thesis are the effects on the endometrium of the Levonorgestrel-releasing Intra-Uterine System (LNG-IUS) and GnRH antagonists. The LNG-IUS is an intrauterine contraceptive device that releases a steady flow of levonorgestrel (20μg daily), a synthetic progestin. The LNG-IUS also has a number of clinical uses other than contraception, such as management of dysmenorrhoea, suppression of menstruation and endometrial protection for women using estrogen replacement therapy (Luukkainen, 2000; Guttinger & Critchley, 2007).

The GnRH antagonist, Cetrorelix, is widely used in in vitro fertilisation (IVF) procedures and causes rapid suppression of LH levels as well as preventing premature LH surges as part of controlled ovarian hyperstimulation (Albano et al., 2000).

Endometrial effects of both of these therapeutic interventions are described in further detail in the following sections.

1.3.1 The Levonorgestrel Intra-Uterine System (LNG-IUS)

Levonorgestrel (LNG) is a potent synthetic progestogen used in a number of contraceptive systems including the Levonorgestrel-releasing Intra-Uterine System (LNG-IUS). Levonorgestrel also has substantial androgenic activity (Kloosterboer et al., 1988) and is an androgenic progestogen. The LNG-IUS is a highly effective contraceptive system that is also used in the management of heavy menstrual bleeding (menorrhagia), as it has been shown to reduce menstrual blood loss (Andersson et al., 1992) and menstrual pain (dysmennorhoea) (Luukkainnen et al., 1990). The LNG-IUS is a small plastic intra-uterine system that is inserted into the uterine cavity and steadily releases levonorgestrel for up to 5 years at a rate of 20μg per 24 hours (Luukkainen, 2000). The LNG is absorbed from the uterus into the systemic circulation; maximum serum levels are achieved within a few hours (Luukkainen, 2000). Following LNG administration proliferation is reduced. Figure 1.12 shows a LNG-IUS.

Figure 1.12 A LNG-IUS

1.3.1.1 Effects of the LNG-IUS on the Endometrium

LNG-IUS usage causes a number of morphological alterations to the endometrium, primarily changes characteristic of decidualisation (Critchley 2003; Guttinger & Critchley 2007). There is a reduction in thickness of the functional layer and the glands become narrow and atrophic. Stromal cells take on the swollen appearance of decidualised stromal cells. Thus, endometrium exposed to constant levonorgestrel delivery is described as pseudo-decidualised endometrium.

These changes can be seen within one month of insertion of the LNG-IUS, but further changes are rare with continued useage. Within 1-3 months of removal of the LNG-IUS, endometrial morphology returns to normal and users return to complete fertility (Andersson et al., 1992; Silverberg et al., 1986).

In addition, an influx of uNK cells, similar to that seen in first trimester decidua, can be observed in LNG-exposed endometrium (Critchley et al., 1998; Critchley, 2003). Increased expression of a number of decidualisation markers such as IGFBP-1, prolactin and prolactin receptor occurs in the endometrium of users of the LNG-IUS (Jones & Critchley, 2000).

Long-term usage of the LNG-IUS is also associated with alterations in sex steroid receptor expression in comparison to normal endometrium. In particular, the progesterone receptor is down-regulated in both the epithelial and stromal cells (Critchley et al., 1998; Critchley, 2003). Estrogen receptor is also down-regulated in

pseudo-decidualised endometrium for at least the first 12 months of LNG-IUS use (Critchley et al., 1998). Expression of the androgen receptor has also been shown to be down-regulated in the endometrium following usage of the LNG-IUS (Burton et al., 2003). The effects of the LNG-IUS on glucocorticoid or mineralocorticoid receptor expression have not previously been reported.

Levonorgestrel can be metabolised by the same enzymes responsible for progesterone and androgen metabolism (primarily 3βHSD, 17βHSD-2, 17βHSD-5 and AKRs; Lemus et al., 1992). The effects of this metabolism may control the availability of LNG for binding to AR and PR (Lemus et al., 1992).

1.3.1.2 Drawbacks of the LNG-IUS

Although the LNG-IUS is a very effective contraceptive and it is also associated with reduced menstrual blood loss and reduced pain, many users report problems with breakthrough bleeding (BTB), i.e. unscheduled bleeding between menstrual periods. This is the most common reason for women to discontinue use of this contraceptive method. Breakthrough bleeding is most common in the first six months of use and often improves with time (Zalel et al., 2003). The mechanisms underlying this breakthrough bleeding have not yet been fully established. There is evidence for increased fragility of blood vesssels, alterations in steroid responsiveness and angiogenic factors playing a role (Milling-Smith & Critchley, 2005; Guttinger & Critchley, 2007). The factors that are altered in the endometrium following LNG-IUS treatment are summarised in Table 1.3.

Table 1.3 Effects of intrauterine LNG on the endometrium.

Morphology	Sex steroid receptors	Local factors	Intracrinology	Other factors
Endometrial atrophy Decidualisation Altered spiral artery formation Superficial vessels dilated and walls thinned	ER ↓ PR ↓ AR ↓	Cytokines ↑ Prostaglandins ↑ VEGF ↑ MMPs ↑ Decidualisation markers ↑	17βHSD-2 ↑ Estradiol ↓ Estrone ↑	Leukocyte infiltration

(Adapted from Guttinger & Critchley, 2007)

Burton et al. (2003) found 17βHSD-2 to be up-regulated in the endometrium in the first three months of LNG-IUS usage, but levels then returned to normal. This would lead to reduced local intracellular estrogen availability, which may contribute to breakthrough bleeding in the first months of use and account for the cessation of breakthrough bleeding after 6 months in the majority of cases. Studies have not, however, been carried out on a number of other steroid metabolising hormones that may work in conjunction with 17βHSD-2 and may be altered in LNG-exposed endometrium.

1.3.2 GnRH antagonists in fertility treatments

A number of technologies are now widely used for the treatment of sub-fertility, including in-vitro fertilisation (IVF) and intra-cytoplasmic sperm injection (ICSI). Both these protocols require ovarian stimulation, carried out using recombinant FSH. Following recombinant FSH administration, premature LH surges are possible, and

ovarian hyperstimulation syndrome can occur. In order to prevent this, it is necessary to modulate gonadotrophin production.

1.3.2.1 GnRH Agonists and Antagonists

Modulation of gonadotrophin production may be achieved with the use of either GnRH agonists or antagonists. GnRH agonists act by facilitating release of large amounts of LH and FSH from the pituitary and causing an up-regulation of GnRH receptor expression, if administered in a pulsatile manner (Lemay et al., 1984). If continuous GnRH agonist is administered, the effect is opposite, the agonist/receptor complex is internalised and receptor expression down-regulated. Thus, gonadotrophin synthesis and release are suppressed, leading to arrest of follicular development and reduction of circulating sex steroid levels. This effect is completely reversible when treatment is stopped. This continuous administration is known in fertility management as a GnRH agonist "long" protocol and it is this which is most commonly used in IVF and considered to be the most effective (Akagbosu, 1999, in Brindsen [ed., 1999]).

GnRH antagonists act by competitively binding GnRH receptors in the pituitary to prevent GnRH action, also leading to the arrest of follicular development. GnRH antagonist protocols allow a shorter treatment cycle and overall reduction in amount of gonadotrophins required (Albano et al., 2000).

Until relatively recently, GnRH agonists were used as the most common method of gonadotrophin suppression in IVF cycles. The "long" protocol however, requires a lengthy treatment period, and additional luteal phase support is required to allow recovery. This is usually achieved with progesterone or human chorionic gonadotrophin (hCG) (Akagbosu, 1999, in Brindsen [ed., 1999]).

The development of GnRH antagonists appeared to have many advantages in overcoming these drawbacks. However, there is much debate about the effect this treatment has on pregnancy rates. Ludwig et al. (2001) found similar rates of pregnancy using GnRH agonist and antagonist protocols, whereas Al-Inany et al. (2006) reported a reduction in pregnancy rates following GnRH antagonist treatment.

1.3.2.2 Effect of treatment on pregnancy rates

Rates of implantation and pregnancy following assisted reproduction techniques remain low despite advances. The reasons for this have not yet been fully elucidated, but there have been reports of an altered steroid receptor expression profile in the early luteal phase (Papanikolau et al., 2005), and also of endometrial advancement following use of GnRH antagonists (Kolibianakis et al., 2002). The window of implantation can be defined as days 5-10 after the LH surge (Sharkey & Smith, 2005). There are currently limited data available on the endometrium during this window, however it is likely to play a major role in determining the success of assisted reproduction techniques. There may be a correlation between endometrial

thickness and success of implantation, although no agreement has been reached on the importance of this factor (Devroey et al., 2004).

Sex steroid hormones are thought to have a vital role in the development of the endometrium prior to implantation (Norwitz et al., 2001; Ma et al., 2003; Lessey, 2002; Apparao et al., 2002). However the precise role these hormones play has not yet been fully defined.

1.4 Aims and Hypotheses

The aims of the studies in this thesis were:

- To investigate steroid metabolism and pre-receptor signalling in human endometrium by studying expression patterns of steroid metabolising enzymes across the menstrual cycle and in the first trimester of pregnancy.
- To investigate the 11βHSD system further by studying the regulation of these enzymes and the receptors by which their products act in endometrial cells *in vitro*.
- To investigate the effects of exogenous steroid administration on steroid metabolism and pre-receptor signalling by studying expression patterns of enzymes in endometrium following clinical treatments.

Thus, the hypotheses were:

- That both glucocorticoid and sex steroid metabolising enzymes are vital in the regulation of the menstrual cycle, and thus expression of these will vary across the menstrual cycle.
- That IL-1α is a regulator of glucocorticoid action and that epithelial cells in the female reproductive tract behave similarly regardless of location.
- That exogenous steroid manipulation causes expression of steroid metabolising enzymes to be perturbed.

Chapter 2:

General Materials and Methods

2.1 Patient Recruitment and Sample Collection

2.1.1 Endometrial samples

Endometrial biopsies were collected from women undergoing surgery or investigation for benign gynaecological conditions. All patients were of reproductive age, had not received exogenous hormones or used an intrauterine contraceptive device in the three months prior to sample collection. All patients, excluding those who gave samples for use in Chapter 4 of this thesis, had regular menstrual cycles of 25-35 days. Cycle length and regularity of the patients studied in Chapter 4 varied, some did not have a discernable cycle at all.

Biopsies were collected in the two ways that follow. Full thickness endometrial biopsies, including both myometrium and basal and functional endometrium were taken after the uterus had been removed by hysterectomy. Endometrium-only (predominantly functional layer) biopsies were sampled vaginally by pipelle suction curette (Laboritoire CCD, Paris, France). All subjects had a blood sample taken at the time of sample collection to determine circulating serum estradiol and progesterone levels by radioimmunoassay (RIA) by Mr Ian Swanston and colleagues, MRC HRSU, Centre for Reproductive Biology, University of Edinburgh. Histological dating was also performed on all samples by Dr. A. Williams, Department of Pathology, Royal Infirmary of Edinburgh according to the criteria of Noyes et al. (1950). In all samples used the circulating serum hormone levels at time

of biopsy collection, histological dating, and patient's reported last menstrual period (LMP) were consistent and used to determine cycle stage.

Two or more biopsies were taken from the same site in the uterus of each patient; or a single biopsy was split into two or more pieces wherever possible to use for both RNA work and immunohistochemistry. Full-thickness biopsies were collected in 4% neutral buffered formalin (NBF; Sigma, Poole, UK) for histology. Endometrium-only biopsies (collected both by pipelle and surgically) were taken in RNAlater storage solution (Ambion, Warrington, UK) and used for RNA extraction, or collected in Dutch-Modification Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma) and used in tissue culture.

2.1.2 Decidua samples

Decidual tissue samples were obtained from women undergoing surgical termination of pregnancy by vacuum aspiration during the first trimester of pregnancy. All procedures were carried out under general anaesthetic. Prior to surgery, patients had an ultrasound scan to confirm viability of the pregnancy and gestational age. All material from the aspiration was collected, and decidual tissue identified by microscopic inspection. Sterile gauze was held over a waste collection vessel and the contents of the tissue collection vessel poured over the gauze. Excess blood and saline were removed by gentle compression, then sterile forceps were used to extract the decidua parietalis. Decidual biopsies were split between NBF and RNAlater solutions as described in 2.1.1.

2.1.3 Informed consent and ethical approval

Written, informed consent was obtained from all patients. Local ethical committee approval was granted for all studies (LREC/05/S1103/14; LREC/1994/6/17; LREC/1702/1993/6/73).

2.2 Tissue Processing

2.2.1 Tissue Processing for RNA Extraction

Tissue for RNA extraction was collected in RNAlater (Ambion) storage solution and transferred to the laboratory. The sample was stored in RNAlater for 24 hours at 4°C, then transferred to a cryotube (Nunc, Roskilde, Denmark) and frozen at -70°C until required for RNA extraction. Samples may be stored in this way for extended periods of time.

2.2.2 Tissue Processing for Histology

Tissue samples for histology were collected in NBF (Sigma) and transferred to the laboratory. Samples were kept at 4°C for 24 hours, then transferred to 70% ethanol and stored at room temperature prior to wax embedding. Processing and wax embedding was carried out in the MRC Human Reproductive Sciences Unit (HRSU) Histology facility. Samples were placed in labelled cassettes in 70% ethanol for automatic processing. In a Leica TP1050 automatic processor, samples underwent dehydration in graded alcohols for 1.5 hours in each alcohol, 2x2 hours in absolute alcohol, then 3x3 hours in xylene, before going through three chambers of molten wax for 1, 1, and 1.5 hours respectively. Following processing, samples were aligned correctly in metal moulds (particularly important for full-thickness biopsies), and molten paraffin wax poured into each mould. The cassette was placed on top of the molten wax and the mould placed on a cooling block to set. When set, the mould

could be gently eased off. Embedded tissue sections were stored at room temperature. When tissue sections were required, the blocks were cooled on ice to make the blocks easier to cut. 5μm sections were cut using a hand-operated microtome (Leica Microsystems, Milton Keynes, UK). Sections were floated on distilled water at 37°C in a heated waterbath and gently picked up by charged coated slides. Sections were dried overnight at 50°C in a drying oven (Lamb RA, East Sussex, UK, model E28.5) then stored at room temperature until required.

2.2.3 Tissue Processing for Tissue Culture

Tissue samples for culture were collected in RPMI medium and stored as close to 37°C as possible prior to transfer to the laboratory. As soon as samples were received in the laboratory the cell separation procedure described in 2.8 was commenced.

2.3 RNA Extraction

RNA was extracted using different methods depending on the type of sample (large and small tissue samples, cells). All RNA samples were treated with DNase to prevent contamination.

2.3.1 RNEasy Midi Kit Extraction

RNA was extracted from tissue samples using the RNEasy Midi Kit (Qiagen, Crawley, UK). Samples were removed from storage and transferred into a large centrifuge tube. β-mercaptoethanol (β-ME; Sigma) was added to the supplied lysis buffer prior to use. 10μl β-ME was added per 1ml lysis buffer to be used. 2ml lysis buffer was added to the tissue and the tissue homogenised using a rotor-stator hand-held homogeniser (Sartorius, London). The tissue lysate was centrifuged at 2300g for 10 minutes in a Thermo IEC CL10 centrifuge and the supernatant transferred to a new centrifuge tube. The pellet was discarded. 70% ethanol (4ml) was added to the lysate and mixed by shaking. The sample was applied to an RNEasy midi column, placed inside a centrifuge tube, then centrifuged at 2300g for 5min. The flow-through was discarded. This stage was performed inside a Class II microbiological safety cabinet.

The procedure was transferred to a fume cupboard. On-column DNase digestion was performed as suggested by the manufacturers as follows. 2ml wash buffer was added to the column and the tube centrifuged for 5 min at 2300g. The flow-through was

discarded. 20µl DNase 1 stock solution (Qiagen) was added to 140µl DNase buffer (Qiagen) per sample and mixed by flicking the tube. The DNase 1 mix was pipetted directly on to the membrane of the RNEasy column and left at room temperature for 15 minutes. A further 2ml wash buffer was added and the column left at room temperature for 5 minutes, then centrifuged for 5 minutes at 2300g. The flow-through was discarded.

2.5ml elution buffer was added to the column, which was then centrifuged for 2minutes at 3000xg and flow-through discarded. A further 2.5ml elution buffer was added to the column, centrifuged for 5 minutes at 2300g and flow-through discarded. The column was transferred to a new tube and 250µl RNase-free water was pipetted directly onto the membrane. The tube was allowed to stand for one minute then centrifuged for 3 minutes at 2300g. The eluate was removed from the tube and passed through the column once more as described to obtain a higher RNA concentration. RNA was then stored at -70°C.

2.3.2 RNEasy Mini Kit Extraction

RNA was extracted from cultured cells using the RNEasy Mini Kit (Qiagen). Culture medium was removed from the cells. Two methods of removing cells from the culture plates were used. In the first method, 1% Trypsin/EDTA (Sigma) was applied as described in 4.2.3, and then the cells were scraped with a pipette tip and transferred to a 2ml tube. These cell suspensions were centrifuged at 2000rpm for 5 minutes, supernatant removed and the pellet re-suspended in lysis buffer+β-

mercaptoethanol from the Qiagen RNeasy mini-kit. Cells were then passed through a 19-gauge needle to homogenise them and frozen at -70°C for later RNA extraction. After the initial experiments it was clear that the yield of RNA was not as great as would be expected, so an alternative method of cell harvesting was sought. This involved washing the cells in PBS and pipetting lysis buffer containing β-mercaptoethanol directly onto the culture plates, before scraping the cells and pipetting them up and down repeatedly. The suspension was transferred to a 2ml tube, passed through a 19-guage needle and frozen at -70°C as before. A greater yield of RNA was obtained using this method. Cell lysates were removed from storage and thawed in a waterbath at 37°C for 20 minutes prior to beginning extraction.

70% ethanol (700μl) was added to the lysate and mixed by pipetting. The sample was applied to an RNEasy midi column, placed inside a centrifuge tube then centrifuged at 10,000rpm in a microcentrifuge for 15 seconds. The flow-through was discarded.

On-column DNase digestion was performed. 350μl lysis buffer was added to the column and the tube centrifuged for 5 minutes at 3000xg. The flow-through was discarded. 10μl DNase I stock solution (Qiagen) was added to 70μl DNase buffer (Qiagen) per sample and mixed by flicking the tube. The DNase 1 mix was pipetted directly onto the membrane of the RNEasy column and left at room temperature for 15 minutes. A further 350μl wash buffer was added then centrifuged for 15 seconds at 10000rpm. The flow-through was discarded.

500µl elution buffer was added to the column, centrifuged for 15 seconds at 1000rpm and flow-through discarded. A further 500µl elution buffer was added to the column, centrifuged for 2 minutes at 10000rpm, and flow-through discarded. The column was transferred to a new tube and centrifuged for a minute at full speed to dry the column. The column was once again transferred to a new tube and 250µl RNase-free water was pipetted directly onto the membrane. The tube was centrifuged for 1 minute at 10000rpm. The eluate was removed from the tube and passed through the column once more as described to obtain a higher RNA concentration. RNA was stored at -70°C.

2.3.3 Trizol Extraction

Some archival RNA samples had been extracted using Trizol (Invitrogen, Paisley, UK) extraction reagent prior to my joining the laboratory. Samples were homogenised in 1ml Trizol RNA extraction reagent (per 100mg tissue) using a hand-held homogeniser and incubated for 5 minutes at room temperature. Homogenates were transferred to a phase lock gel tube (Eppendorf, Cambridge, UK) and 200µl chloroform added. Tubes were shaken for 15 minutes. The aqueous phase was transferred into a clean 2ml tube and 500µl isopropanol added. Samples were incubated for 10 minutes at room temperature, and then centrifuged at 13,000rpm for 10 minutes at 4°C in a Sigma 1-15K centrifuge. The supernatant was removed and the pellet washed in 70% ethanol, and vortexed, then centrifuged at 9000rpm for 10 minutes at 4°C. Supernatant was again removed. The pellet was dried on ice for 5

minutes and re-suspended in 50µl RNA storage buffer. Samples were left on ice for 1 hour to disperse in storage buffer, then stored at -70°C.

When this method was used it was necessary to DNase-treat RNA samples separately prior to use for PCR. This was done using an Ambion DNase treatment kit (Ambion). The volume of RNA required to give a final concentration of 100ng/µl was calculated, and the correct volume of water to make 10µl added. 1µl of buffer and 1µl of DNase were added and the mixes incubated at 37°C in a Hybaid Omn-E PCR machine for 20 minutes. 0.5µl DNase Inactivation Reagent was then added and incubated for 2 minutes at room temperature. Samples were centrifuged for 1 minute at 12,500rpm, then frozen at -70°C.

2.3.4 Measuring RNA concentration and quality

RNA concentration and quality were measured using an Agilent 2100 Bioanalyzer and RNA 6000 Nano Kit (Agilent Technologies, Stockport, UK).

The Agilent 2100 bioanalyzer instrument detects biomolecules by laser-induced fluorescence. During chip preparation a dye concentrate is mixed with a gel. The priming station allows the channels of the chip to be filled with the gel-dye mix. During the chip run the dye intercalates directly with the RNA to be analysed and outputs data in the form of an electropherogram.

Cleaning chips were used to clean the electrodes with both RNase Zap (Ambion) and nuclease-free water (Sigma) prior to use. Reagents were removed from the fridge 30 minutes before use.

Gel was prepared by spinning the RNA 600 Nano gel matrix at 4000rpm for 10 minutes in a spin filter. The gel was stored at 4°C for later use. Prior to use, dye containing DMSO was added to the gel to allow it to bind to nucleic acids. An RNA Nano chip was placed in the priming station and gel-dye mix pipetted into the appropriate well. The chip priming station was closed and the plunger pressed down for 30 seconds to pressurise the gel. RNA 6000 Nano marker was pipetted into the ladder well and each of the sample wells. Any wells which were not to be used received 6μl of dye instead of 5μl. The RNA 6000 ladder was denatured at 70°C for 2 minutes and pipetted into the ladder well. 1μl of each sample was loaded into each sample well on the chip. The chip was vortexed for 1 minute and inserted into the Agilent 21000 bioanalyser and the run started. Results were presented in electropherograms. An example of the ladder electropherogram is shown in Figure 2.1. Figure 2.2 shows an electropherogram of a good and a poor quality RNA sample.

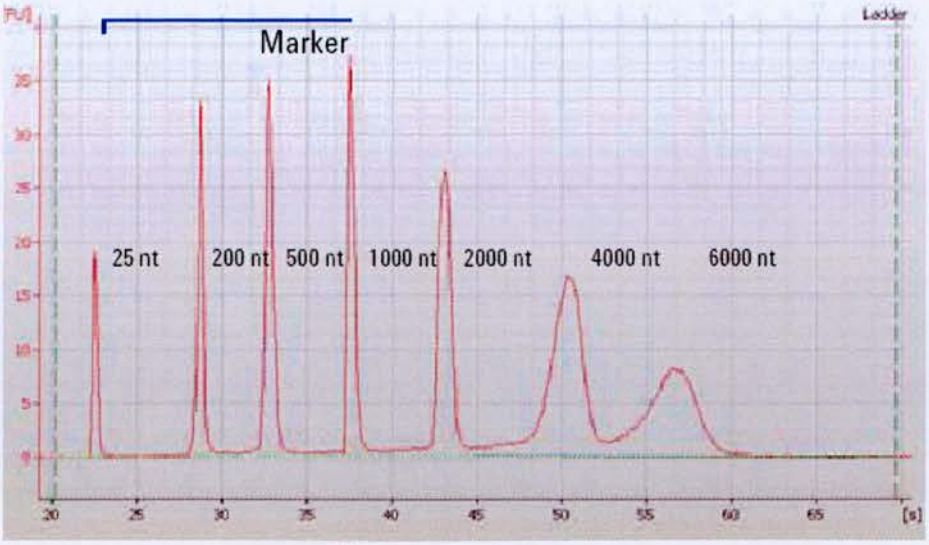


Figure 2.1 Ladder Electropherogram from RNA 6000 Nano analysis.Taken from Agilent 2100 Bioanalyzer brochure, Agilent Technologies.

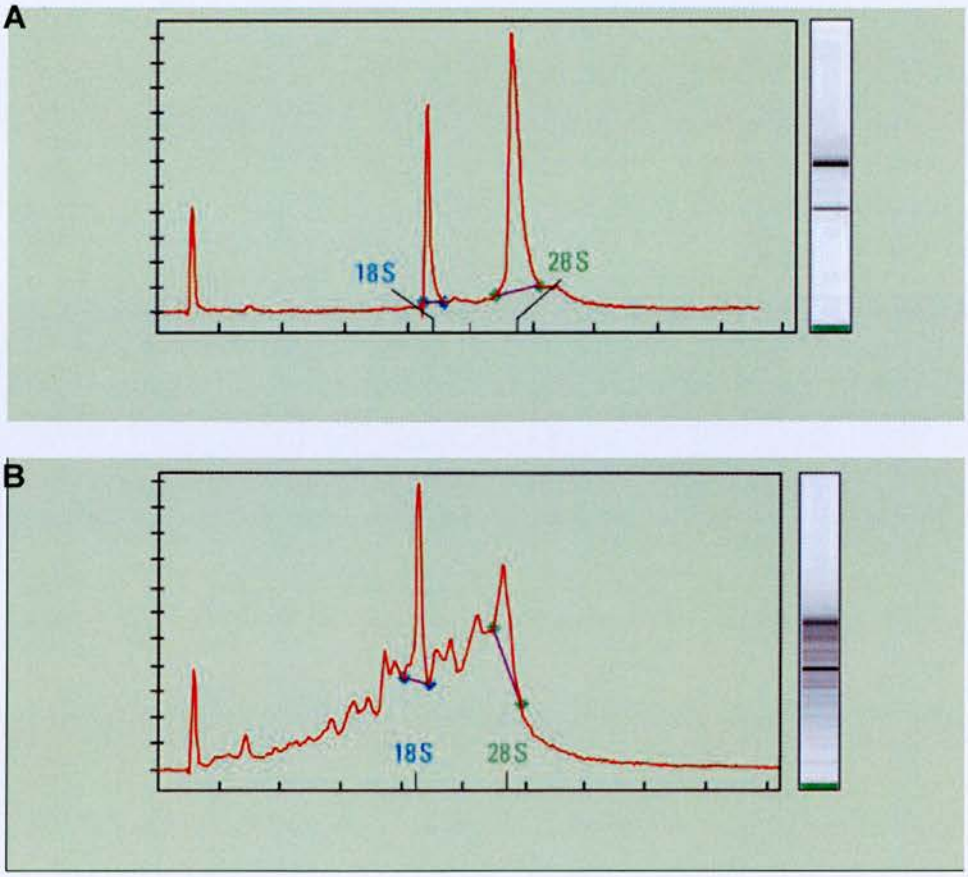


Figure 2.2 Examples of an electropherogram produced from a good (A) and poor (B) quality RNA samples. The good quality sample has two distinct 18S and 28S peaks, however in the poor quality sample the peaks are less distinct. Taken from Agilent 2100 Bioanalyzer brochure, Agilent Technologies.

RNA quality was further tested by the determination of an RNA Integrity Number (RIN) by the 2100 Expert software (Agilent Technologies). Intact RNA gives a RIN of 10, a RIN below 7 suggests there is degradation of the RNA. For QRT-PCR, all samples had a RIN of 8.5 or above. When RNA concentration was determined, if it was greater than 100ng/μl, the RNA was diluted in nuclease-free water to100ng/μl and stored in aliquots at -70°C.

2.4 Reverse-Transcriptase PCR

RNA was reverse-transcribed using a Multiscribe reverse transcriptase (1.25IU/μl), random hexamers (2.5 μmol/l), magnesium chloride (5.5 mmol/l), dNTPs (1mmol/l) and an RNase inhibitor (0.4 IU/μl) (all PE Applied Biosystems, Warrington, UK). Reactions consisted of 8μl of reaction mix and 2μl RNA (100ng/μl) or multiples thereof. Reaction mixes were thoroughly vortexed and briefly centrifuged in a microcentrifuge to ensure all contents were in the bottom of the tube. Tubes were then put in a thermal cycler (Omn-E, Hybaid, UK) and samples were incubated for 25°C for 20 minutes, 42°C for 60 minutes, then 95°C for 5 minutes. Two negative controls were also included, an "RT-negative" containing no Multiscribe RTase and an "RT-water" containing nuclease-free water in place of RNA. The resulting cDNA was stored at -20°C.

2.5 Quantitative Real-Time PCR (Taqman)

Quantitative Real-Time PCR (QRT-PCR) measures levels of a gene-specific sequence of cDNA generated in a reverse-transcriptase PCR reaction, and thus provides a measure of RNA expression in a tissue. QRT-PCR was carried out using the Taqman system and ABI Prism 7900HT sequence detection system (both PE Applied Biosystems). This works by producing a fluorescent signal during the amplification of the PCR product. Forward and reverse primers are designed for a target DNA sequence and the probe to anneal to this target sequence between the primers. The probe has two labels, a reporter label at the 5' end (FAM; 6-carboxyfluoresceine) and a quencher at the 3' end (TAMRA; 6-carboxytetramethylrhodamine). The probe is designed to have a higher melting point than the primers. When intact, the quencher suppresses the fluorescence of the reporter. The probe anneals to the cDNA during the polymerisation step then amplification occurs. The probe is cleaved during this step by AmpliTaq Gold (within Taqman Universal PCR Master Mix, PE Applied Biosystems) and the reporter and quencher are separated. Fluorescence can now be detected. The amount of fluorescence is directly proportional to the amount of PCR product, and non-specific amplification does not cause fluorescence. This is represented diagrammatically in Figure 2.3. 18S ribosomal RNA detection is used as an internal control in this system. The 18S probe also has a reporter (VIC) which emits fluorescence at a different wavelength to the FAM reporter on the gene of interest probe. Quantification is relative to 18S as levels remain relatively constant in cells. Data are acquired when PCR amplification is in the exponential phase. The data are

in the form of the cycle number where the fluorescence of the reporter dye rises above a threshold, this value is the threshold cycle, CT.

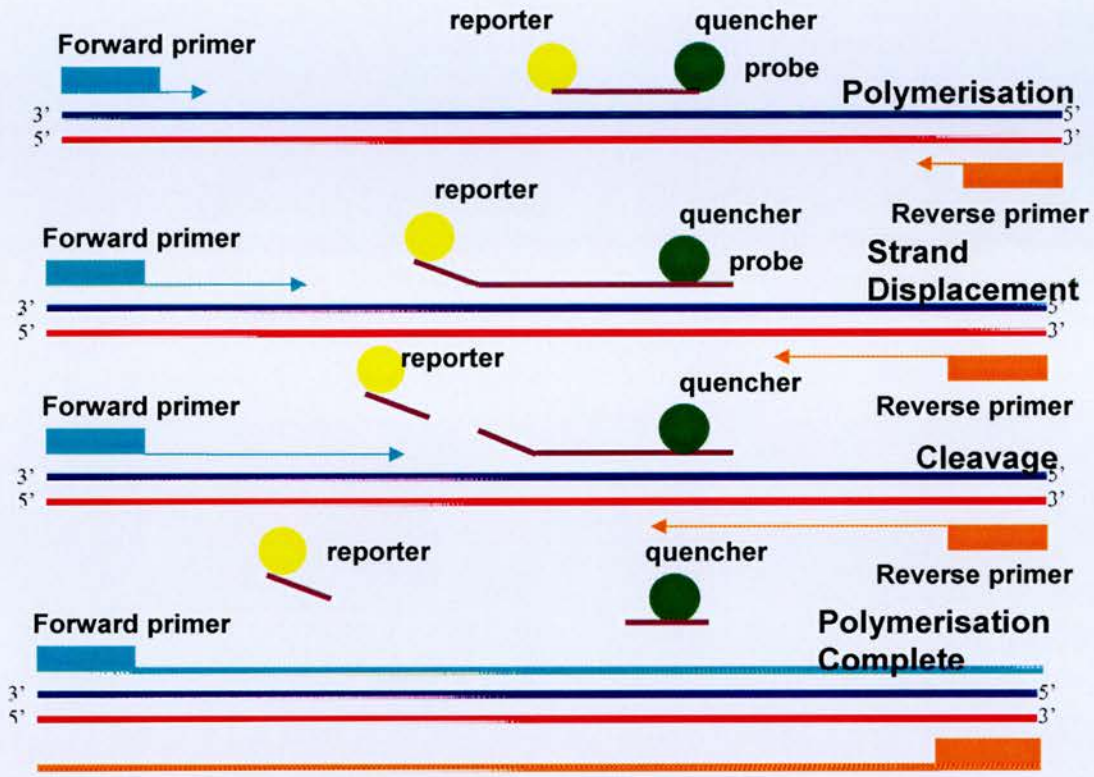


Figure 2.3 Diagrammatic representation of Taqman QRT-PCR. Adapted from Taqman PCR Reagent Kit Protocol.

2.5.1 QRT-PCR

Taqman primers and probes were either designed in-house using Primer Express software, or purchased from Assay on Demand (PE Applied Biosystems). Details of primers and probes used are given in the relevant chapters. Primers designed in-house were manufactured by Biosource (Nivelles, Belgium) or Eurogentec (Southampton, UK) and diluted to 250nM and probes to 50nM in nuclease-free water

(Sigma). Assay on Demand primer/probe sets were used neat as they are provided pre-optimised at a final concentration of 900nM primer and 250μM probe. Reaction mixtures containing TaqMan Universal PCR Master Mix (PE Applied Biosystems; consists of AmpliTaq Gold® DNA Polymerase, AmpErase® UNG, dNTPs with dUTP, Passive Reference 1, and optimized buffer components) and specific forward and reverse primers and probe were made in a final volume of 25μl per well. Ribosomal 18S primers and probe (PE Applied Biosystems) were added at a final concentration of 50nM. Samples were run in triplicate on an ABI Prism 7900 sequence detection system (PE Applied Biosystems). For each sample, a tube containing 72μl of the reaction mixture and 3μl cDNA was prepared. 24μl of this was pipetted into three separate wells on a 96-well plate. The RT-negative and RT-water controls were included, alongside a "no template" control, where nuclease-free water was included instead of cDNA.

2.5.2 Analysis of output

Data were output in the form of "CT" values; the number of PCR cycles required before there is sufficient cDNA amplification to be detected. A CT value was given for both the gene of interest and 18S. These data were transferred to a Microsoft Excel spreadsheet for analysis. A number of calculations were performed on the data. The first was calculation of the ΔCT value, by subtracting the 18S CT value from the CT of the gene of interest. Then the average ΔCT was calculated as the mean of the 3 CT values for each sample. To calculate ΔΔCT, a comparator sample had to be chosen. For experiments looking at samples across the menstrual cycle, this was a

proliferative phase sample; the same sample was used in each experiment wherever possible to ensure all experiments were comparable. For other experiments, the comparator was an untreated control sample. The ΔΔCT was calculated by subtracting the ΔCT of the comparator from the ΔCT of each sample. The final calculation was to find (2^- ΔΔCT) for each sample, to give a fold increase in expression over comparator. The mean fold increase for each sample group was then calculated. An example spreadsheet is shown in Figure 2.4.

CT(sample)-CT(18S)

average of the 3 triplicates for each sample.

average-comparator average

=2^(-DDCT)

Sample	CT(18S)	CT(sample	DCT	AVERAGE	DDCT	2^-DDCT
1	11.3966	29.13059	17.73399	17.74	-0.03	1.02346
1	11.10005	29.08031	17.98026			
1	11.83673	29.33213	17.4954			
2	11.28214	28.79379	17.51166	17.61	-0.16	1.1204
2	11.42452	28.96353	17.53901			
2	11.00313	28.77044	17.7673			
3	11.63524	27.03788	15.40265	14.92	-2.85	7.20317
3	11.88735	26.58823	14.70089			
3	11.71255	26.37313	14.66058			
4	11.30327	25.67604	14.37276	14.21	-3.56	11.7859
4	11.31162	25.58229	14.27068			
4	11.16155	25.15116	13.98961			

Figure 2.4 Example Microsoft Excel Spreadsheet showing Taqman data analysis

2.5.3 Statistical analysis

Statistical analysis of QRT-PCR data was performed using one-way analysis of variance and Dunn's post-hoc test, using GraphPad InStat software. The significance criterion was set at $p<0.05$.

2.6 Immunohistochemistry

Expression patterns and localisation of proteins in the endometrium were investigated by immunohistochemistry using a number of specific primary antibodies. A standard avidin-biotin horseradish peroxidase (ABC-HRP) method was employed. Briefly, the primary antibody binds specifically to the protein of interest, and the biotinylated secondary recognises and binds to antigenic sites on the primary antibody. An avidin-biotin-HRP complex binds to the biotin on the secondary antibody and DAB binds the HRP and is oxidised. This causes a visible colour change which results in the brown staining of tissue sections where the antibody has bound. Figure 2.5 shows this in diagrammatic form.

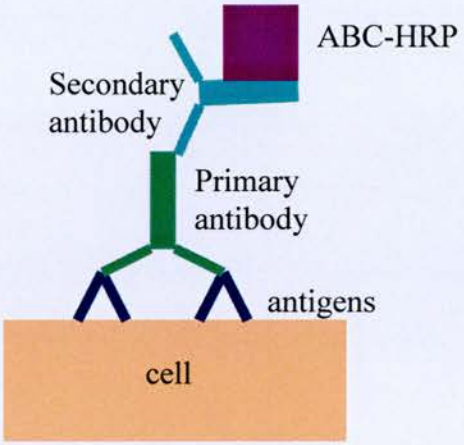


Figure 2.5 Diagrammatic representation of ABC-HRP immunohistochemistry method. Adapted from Dako UK Ltd. Handbook of Immunochemical staining methods 3rd edition, Boenisch et al., 2001)

2.6.1 Slide Preparation

Cut tissue sections were appropriately labelled, dewaxed for 10 minutes in xylene, then dehydrated in graded alcohols for 2 minutes each, all in a fume cupboard. Slides were washed in distilled water for 10 minutes.

2.6.2 Antigen Retrieval

To break the bonds created by fixation and allow antibodies to bind, antigen retrieval was required for use of some antibodies. This was done by pressure cooking in 0.01M citrate buffer (pH6). 2l of buffer was placed inside a pressure cooker on a hot plate and allowed to heat. When the buffer was almost boiling, the slides were gently placed inside the pressure cooker, ensuring they were completely immersed in buffer and the lid sealed. When full pressure was reached, the slides were heated for 5 minutes, then the pressure released. When all the pressure was released, the lid was removed and the pressure cooker allowed to cool for 20 minutes to prevent the sections coming off the slides when they were removed from the buffer. Following cooling, sections were washed in PBS (Sigma) for 10 minutes.

2.6.3 Endogenous Peroxidase Block

Endogenous peroxidases have to be blocked to prevent excess background staining. A 3% hydrogen peroxide solution (VWR, Leicestershire, UK) was made by diluting stock hydrogen peroxidase (VWR) in distilled water. Slides were immersed in the

solution for 10 minutes at room temperature, then washed twice for 5 minutes in PBS whilst being gently agitated.

2.6.4 Avidin/Biotin Block

Endogenous avidin and biotin also need to be blocked to prevent excess background staining. An avidin-biotin blocking kit (Vector, Peterborough, UK) was used. Slides were placed in a humidified chamber and avidin applied, then slides were incubated for 15 minutes. Concentrations of avidin and biotin solutions are not available. Slides were briefly washed in PBS while agitated for 2 minutes and returned to the humidified chamber where biotin was applied. Slides were incubated at room temperature for a further 15 minutes, then washed again in PBS.

2.6.5 Non-immune block

Background staining was further prevented by the inclusion of a non-immune block to prevent non-specific binding of the secondary antibody. A solution of serum was prepared by diluting the serum of the species the secondary antibody was raised in 1:5 in PBS and adding 5% BSA (Sigma). This solution was applied to slides in a humidified chamber and incubated for 20 minutes at room temperature. Sections were not washed following this procedure.

2.6.6 Primary antibodies

Primary antibodies were diluted to the appropriate concentration determined by titration in the serum used for blocking. The primary antibodies used and the working dilutions are shown in the relevant chapters. Where possible, the primary antibody was pre-absorbed against a blocking peptide for use as a negative control. Alternatively, pre-immune serum or generic immunoglobulins from the same species at the same concentration as the primary antibody was used. Positive controls were tissue sections known to express high levels of the protein of interest. Details of specific positive and negative controls are given in the appropriate chapters. Primary antibodies and negative controls were applied to sections in a humidified chamber and covered with coverslips to prevent drying. Sections were incubated overnight (approx. 18 hours) at 4°C. Following incubations, sections were dipped in PBS to gently remove coverslips, then washed in PBS containing 0.05% Tween-20 (PBST; Sigma) with gentle agitation.

2.6.7 Secondary antibodies

Biotinylated secondary antibodies raised against the immunoglobulins of the species in which the primary antibody was raised were diluted as described in the appropriate chapters in the serum solutions used for the non-immune block. The antibody was applied to sections in a humidified chamber and incubated for 1 hour at room temperature. Following incubation slides were washed in PBST for 10 minutes with gentle agitation.

2.6.8 Tertiary antibody: ABC-Elite

The ABC-Elite detection system (Vector) was used to amplify the signal from the biotinylated secondary antibody. The ABC-Elite was prepared by adding 1 drop of solution A and 1 drop of solution B from the kit to 2.5ml PBS, 30 minutes prior to use and kept at room temperature. The solution was added to the slides in a humidified chamber and they were incubated for 1 hour at room temperature, then washed for 10 minutes in PBST with gentle agitation.

2.6.9 Developing and counterstaining

Slides were developed and staining visualised using 3,3'-diaminobenzidine (DAB; Dako, Cambridge, UK). 1 drop of chromagen (concentration of DAB stock unavailable) was added to each ml of buffer solution required and DAB dropped onto sections in a humidified chamber, with care taken that all sections were incubated for equivalent time. When brown staining was visualised, DAB was washed off with plenty of distilled water and slides washed for at least 10 minutes in distilled water with gentle agitation. Sections were then counterstained in Harris' haemotoxylin (Pioneer Research Chemicals Ltd, Colchester, UK) for 30 seconds and nuclear staining developed in Scott's tap water (Pioneer Research Chemicals Ltd.) for 10 seconds. Slides were then dehydrated in graded alcohols and cleared in histoclear (Pioneer Research Chemicals) and xylene for 10 minutes each before mounting under a borosilicate glass coverslip (VWR) using Pertex mounting medium (Cellpath plc, Hemel Hempstead, UK).

2.6.10 Visual analysis

Staining was analysed semi-quantitatively and blindly by two observers, myself and another member of the lab team, using a 4-point scoring system where 0=no staining, 1=mild staining, 2=moderate staining and 3=intense staining. Both layers of the endometrium were analysed separately where possible and glands, surface epithelium, stroma and vessels were analysed over the whole section. Where relevant, notes were made as to the location of vascular staining. Scores from each observer were compared, areas of disagreement were re-analysed and a final score reached. Generally the two observers' scored had a good degree of correlation. Mean scores were then calculated for each group of tissue sections.

2.6.11 Statistical analysis

Basic statistical analysis was performed using InStat GraphPad software, by the non-parametric Kruskall-Wallis test, followed by Dunn's post-hoc multiple comparison test. The criterion for significance was set at $p<0.05$.

2.6.12 Photomicroscopy

Tissue sections were photographed using an Olympus Provis microscope (Olympus Optical Co., London, UK) and Kodak DCs330 camera (Eastman Kodak Co., Hemel Hempsted, UK). All images were photographed at 20x magnification unless

otherwise stated. Images were re-sized and compiled using Photoshop computer software (Adobe Systems, San Jose, California, USA).

2.7 Western Blotting

Specificity and quality of previously untested antibodies was confirmed using standard western blotting techniques. Briefly, homogenised tissue samples were mixed with a bromophenol blue dye and run on a gel using electrophoresis. The proteins in the gel were then transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore, Watford, UK) using electrotransfer, then probed with primary antibody, to which antibodies on an HRP-conjugated secondary antibody bind. Protein expression was visualised using chemiluminescent substrate, which reacts with HRP to emit light.

2.7.1 Sample preparation

Tissue samples were homogenised in a buffer consisting of 10mM Tris, 0.3M sucrose and 1mM EDTA at pH7.4 using a hand-held electric homogeniser. Homogenates were centrifuged at 1500rpm in Beckmann centrifuge. Sample concentration was measured by Bradford Assay and samples were mixed 2:1 with loading buffer consisting of SDS, glycerol, β-mercaptoethanol and bromophenol blue for visualisation purposes.

2.7.2 Electrophoresis

A 10% resolving gel was prepared consisting of water, acrylamide, 1.5M Tris pH8.8, 10% SDS solution, 10% ammonium persulphate solution (AMPS) and TEMED. The

gel was poured into gel apparatus and allowed to set. A 10% stacking gel was then prepared and poured on top of the resolving gel, combs inserted to create wells and allowed to set. Gels were not denatured. The gels were then transferred to an electrophoresis tank containing electrode running buffer (Tris, glycine, SDS) and samples loaded. 20μg of each protein sample was loaded. Alongside the samples Precision Plus Protein Standards, all blue, (Bio-Rad, Hemel Hempstead, UK) were run for identification purposes. The electrophoresis tank was connected to a power supply and run at 200V, 50mA, 10W until the dye had reached the end of the gel, and then removed. The gel was washed in transfer buffer (Tris, glycine) before electrotransfer.

2.7.3 Electrotransfer

The protein was transferred from the gels onto PVDF membranes which had been previously washed in methanol. The gel and membrane were assembled in electrotransfer apparatus and connected to a power pack. The transfer was performed at 400V, 250mA, 100W for approximately 90 minutes (longer for larger proteins).

2.7.4 Western Blot

The membranes were then blocked for 2 hours in a 3% powdered milk solution in PBS containing 0.05% Tween-20 (Sigma; PBST) at 4°C. A solution of primary antibody was made up in the milk solution; dilutions are detailed in the appropriate chapters. Following blocking, the membranes were washed 3 times for 10 minutes in

PBST and incubated with primary antibody at dilutions given in the appropriate chapters overnight at 4°C. Membranes were again washed 3 times for 10 minutes in PBST and the secondary antibody applied. This was HRP-conjugated and directed against immunoglobulins of the species in which the primary antibody was raised, made up in milk solution. The membranes were incubated for 1 hour at room temperature in secondary antibody then washed 6 times for 10 minutes in PBST. The blots were developed by chemiluminescence (ECL plus kit, Amersham Life Sciences, Amersham, UK). The chemiluminescent substrate reacts with the HRP bound to the secondary antibody the protein is detected by light emission. The ECL reagent was applied for 5 minutes at room temperature, then the blots developed using a Typhoon 9400 phosphorimager (Amersham Life Sciences). Molecular size of the bands was determined by comparison with the protein standards.

2.8 Cell Separation and Culture

Samples were collected in Dutch Modification RPMI culture medium (Sigma). These biopsies were then washed in Dulbecco's PBS (Sigma) and finely diced using a sterile scalpel. When tissue was diced, 2ml PBS was added, with 200ul collagenase (10ng/μl; Sigma) and 20μl DNase (100ng/μl; Sigma). The tissue was then transferred in this suspension to a sterile 15ml tube and incubated at 37°C for 2 hours and 15 minutes. Following this incubation, 2ml culture medium (F medium) consisting of Dutch modification RPMI 1640 supplemented with 10% fetal calf serum, penicillin (50μg/ml), streptomycin (50μg/ml), L-glutamine (2mmol/l) and gentamycin (5μg/ml) (all Sigma) was added to stop the digestion reaction. The solution containing the digested tissue was transferred to a sterile Petri dish. It was then passed repeatedly through a 19G needle (at least 6 times, until no large lumps remain), then filtered through a 70μm filter, then again through a 40μm filter. Each filter was washed into a clean Petri dish with Dulbecco's PBS (Sigma) when the filtrate had passed through. The filtrate was then centrifuged at 1700rpm for 3 minutes (Thermo IEC CL10 centrifuge) and the supernatant discarded. The pellet was re-suspended in 10ml serum-containing "F" media and transferred to a $75cm^3$ culture flask. These cells were the stromal component and were cultured at 37°C until confluent (1-2 weeks, $1.2x10^6$ cells), with media changed every 7 days.

The fragments of tissue that were washed out of the filters were transferred to a sterile 15ml tube with 400μl 10x collagenase and 40μl 100x DNase and incubated at 37°C for a further 2 hours. Following this incubation, 4ml culture medium (HOSE 1)

consisting of Medium199:MCDB105 (1:1 vol/vol) supplemented with fetal calf serum (15% vol/vol), streptomycin (50 µg/ml), penicillin (50 IU/ml) and L-glutamine (2 mmol/litre) (all Invitrogen) was added to stop the reaction. The suspension was passed repeatedly through a 19G needle as before, then centrifuged at 1700rpm for 3 minutes. The supernatant was discarded and the pellet re-suspended in 10ml HOSE 1 medium (as used routinely in the laboratory for ovarian surface epithelial cells), before being transferred to a $75cm^3$ culture flask. These cells were epithelially enriched, and were cultured until confluent (3-4 weeks; $1.2x10^6$ cells) in the same manner as the stromal cells. The presence of epithelial cells in the cultures was confirmed visually. Figure 2.6 shows typical endometrial epithelial cells in culture, and Figure 2.7 shows stromal cells.

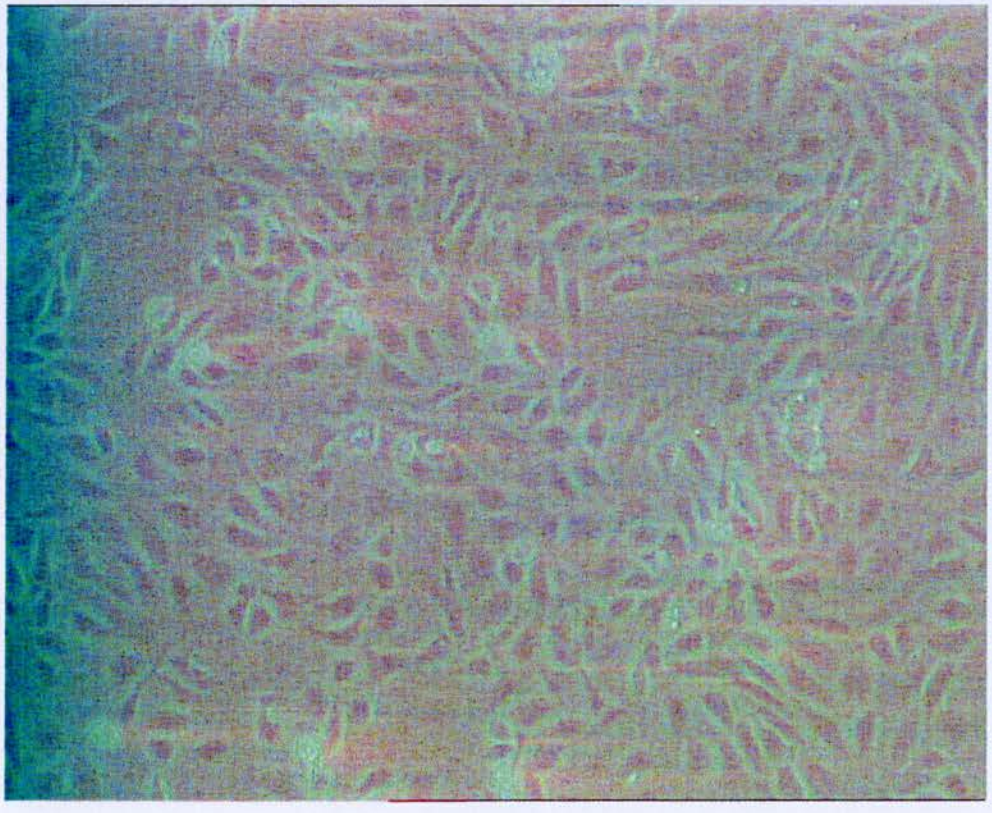


Figure 2.6 Representative sample of endometrial epithelial cells in culture. Images taken at 20x magnification.

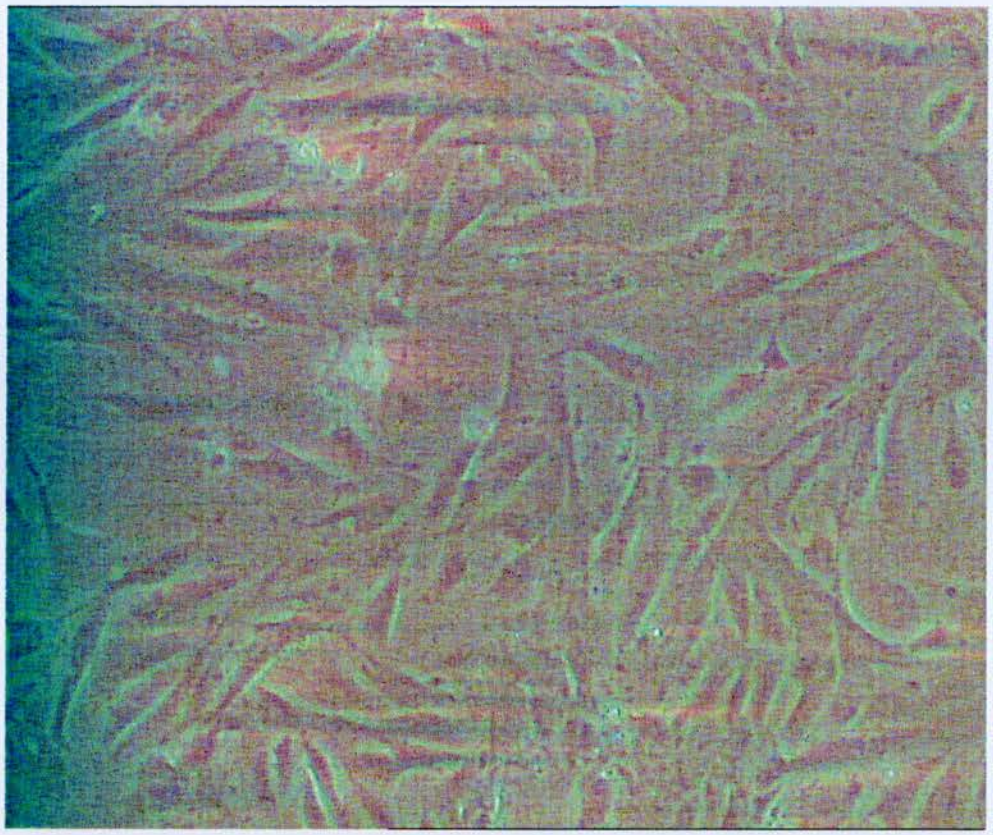


Figure 2.7 Representative sample of endometrial stromal cells in culture. Images taken at x20 magnification.

Additional samples of cells from the surface of the endometrium were collected (N= 7) using a "Tao" brush (Cook, Limerick, Ireland) which had been inserted into the uterine cavity via the cervix/endocervical canal and rotated to sweep the endometrial surface. The brush was then shaken into a tube of HOSE 1 media to remove the scrapings, which were transferred to a $75cm^3$ culture flask and cultured as for the other cell types. These cells were noted to be a mix of stromal and epithelial cells.

Chapter 3:

Glucocorticoid metabolism and pre-receptor signalling in the human endometrium

3.1 Introduction

Ovulation, implantation and menstruation are pivotal reproductive events, all of which involve extensive tissue remodelling and subsequent repair. A number of factors mediate this remodelling and these factors are themselves regulated by steroids and other locally produced mediators including cytokines. Steroids involved in the reproductive system include androgens, estrogens, progestins and glucocorticoids. Pre-receptor signalling events and metabolism associated with these steroids are likely to play important roles in the regulation of tissue remodelling.

Glucocorticoids are proposed to have multiple roles in the human endometrium across the menstrual cycle, in events involving tissue remodelling such as menstruation and implantation. It is now accepted that menstruation is an inflammatory process as first proposed by Finn (1986), and that glucocorticoids are likely involved in modulating this response (Henderson et al., 2003).

Glucocorticoid action is regulated at a local, tissue-specific level by the 11βHSD enzymes. 11βHSD-1 predominantly converts cortisone to active cortisol in intact cells, whereas 11βHSD-2 is a unidirectional enzyme that converts cortisol to cortisone. Cortisol acts by binding to its cognate receptor, the glucocorticoid receptor (GR) and activating an anti-inflammatory response. Cortisol also binds with high affinity to the mineralocorticoid receptor (MR); however, activation of the mineralocorticoid receptor can lead to clinical disorders such as apparent mineralocorticoid excess (AME). Thus the balance of 11βHSD-1 and -2 is important

to regulate cortisol metabolism and action. Consequently, 11βHSD-1 tends to be the predominant enzyme found in cortisol target tissues expressing GR, while 11βHSD-2 can be found co-localised with MR in mineralocorticoid target tissues (Driver et al., 2001; Hirasawa et al., 2000).

11βHSD expression in the endometrium has been studied previously by Smith et al. (1997), who found that both enzymes were active across the cycle. Only 11βHSD-2 protein has been studied in the endometrium due to the lack of a suitable antibody for localisation of 11βHSD-1. GR expression has been studied across the cycle by Bamberger et al. (2001) and Henderson et al. (2003), who found it to be expressed across the menstrual cycle and localised to stromal and endothelial cells of endometrium and additionally in glandular epithelia of first trimester decidua (Henderson et al., 2003). There are no reports currently of MR expression patterns in human endometrium.

Uterine natural killer (uNK) cells have been shown to express GR (Henderson et al., 2003), and earlier reports have shown glucocorticoids to act on these cells. However there is little known about the expression of 11βHSD-1 and -2, and MR in this phenotypically unique group of cells.

The aims of this chapter are to study the expression patterns of 11βHSD-1, -2, GR and MR in human endometrium across the menstrual cycle, in first trimester decidua and in uNK cells at both mRNA and protein levels.

3.2 Materials and Methods

3.2.1 Tissue Collection and Subjects

Local ethical approval and informed consent were provided by patients for all endometrial tissue collected. Tissue samples were collected as described in 2.1.1. Briefly, 18 biopsies consisting primarily of the functional layer of the endometrium were collected at hysterectomy or by pipelle sampling device and used for RNA extraction. Five first trimester decidua biopsies were collected at elective surgical termination of pregnancy and used for RNA extraction.

34 full-thickness endometrial biopsies were collected at hysterectomy and wax-embedded for use in immunohistochemistry. Five first trimester decidua biopsies were collected at elective surgical termination of pregnancy and also wax embedded for immunohistochemistry.

All subjects were of reproductive age, with regular menstrual cycles of 25-35 days and had no exogenous hormone treatment in the previous 3 months. Tissue samples were consistent across three parameters: date of reported last menstrual period, histological stage and ovarian stage of cycle as determined by serum estradiol and progesterone concentrations at the time of biopsy, measured by RIA, to allow for variation from the classic 28 day cycle.

Details of these samples are shown in Tables 3.1 and 3.2.

Table 3.1 Endometrial samples used for QRT-PCR (functional layer only)

Sample	E2 level at time of biopsy (pmol/l)	P4 level at time of biopsy (nmol/l)	Histological stage
1	118	2.9	Menstrual
2	151	4.3	Menstrual
3	291	2.9	Menstrual
4	167	1.6	Menstrual
5	423	5.2	Proliferative
6	534	3.4	Late proliferative
7	842	7.1	Mid proliferative
8	544	2.3	Mid proliferative
9	604	42.5	Early secretory
10	368	12.9	Early secretory
11	574	14.4	Early secretory
12	534	39.2	Early secretory
13	276	42.1	Mid secretory
14	552	78.9	Mid secretory
15	680	106.2	Early secretory
16	129	5.7	Late secretory
17	602	14.7	Late secretory
18	490	22.9	Mid-late secretory

Note: A sub-set of these samples were used for each experiment, n=4 or 5 for each stage. Some archival samples were used up in the course of the experiments so had to be substituted with newer samples.

Table 3.2 Endometrial samples used for immunohistochemistry (full thickness biopsies)

Sample	E2 level at time of biopsy (pmol/l)	P4 level at time of biopsy (nmol/l)	Histological date
1	371	7.5	Menstrual
2	223	3.0	Menstrual
3	*	*	Menstrual
4	*	*	Menstrual
5	169	2.8	Proliferative
6	1233	2.6	Late proliferative
7	642	1.6	Late proliferative
8	43	4.0	Early proliferative
9	234	1.2	Early proliferative
10	762	1.1	Mid proliferative
11	681	3.8	Late proliferative
12	694	2.3	Early proliferative
13	808	3.7	Mid proliferative
14	847	5.3	Late proliferative
15	207	12.2	Early secretory
16	124	19.1	Early secretory
17	282	23	Early secretory
18	534	39	Early secretory
19	202	17	Early secretory
21	853	101	Mid secretory
22	265	39.4	Mid secretory
23	404	65	Mid secretory
24	496	31.1	Mid secretory
25	218	42.3	Secretory
26	120	71.3	Secretory
27	369	12.2	Late secretory
28	492	2.6	Late secretory
29	189	4.1	Late secretory
30	191	8.6	Late secretory
31	129	5.7	Late secretory
32	819	6.4	Late secretory
33	275	5.3	Late secretory
34	176	11.3	Late secretory

* archival samples, data not available.

Note: A sub-set of these samples were used for each experiment, n=4 or 5 for each stage. Some archival samples were used up in the course of the experiments so had to be substituted with newer samples.

The following samples of first trimester decidua were used for both QRT-PCR and immunohistochemistry.

Table 3.3 Decidua samples used

Sample	Gestation (weeks+days)
1	8+6
2	7+4
3	<8
4	8+2
5	8+5

Uterine natural killer cells were isolated by Dr Ashley Moffett, as previously described (Henderson et al., 2003). Briefly, 1x 10^8 decidual cells were suspended in 300 μl buffer (PBS/2 mM EDTA/1% human serum). After the addition of 0.5% human γ-globulins in PBS and 100 μl CD56 magnetic cell sorting microbeads (Miltenyl Biotech, Bergisch Gladbach, Germany), the suspension was incubated at 4 C for 20 min. The cells were washed, resuspended in buffer, and applied to a VarioMACS magnet (Miltenyl Biotech). The column was washed, and the $CD56^+$ cells were eluted and resuspended in RPMI/10% fetal calf serum. The purity of the decidual NK cells was greater than 97%, as confirmed by flow cytometry.

3.2.2 RNA extraction and reverse-transcriptase PCR

RNA extraction and reverse-transcriptase PCR were performed as described in sections 2.3 and 2.4.

3.2.3 Taqman Quantitative Real-Time PCR

QRT-PCR was performed using standard methods as described in Chapter 2. Oligonucleotide forward and reverse primers and oligonucleotide Taqman probes were used to detect the sequences of interest. The probes used were designed using Primer Express software as previously described (11βHSD-1, -2, GR) (Rae et al., 2004a, Henderson et al., 2003), or commercially available from PE Applied Biosystems Assay on Demand service (MR). The following specific primers and probes were used:

Table 3.4 Taqman Primers and Probes

11βHSD-1 forward	AAG ATG TTC CTG CAT GGA TTT C
11βHSD-1 reverse	AGC TCT GCG CCA AGA AGA AGT
11βHSD-1 probe	TGA CAG CTC ACT CTG GAC CAC TCT TCT GA
11βHSD-2 forward	GGC CAA GGT TTC CCA GTG A
11βHSD-2 reverse	GTT GTG CCA GGA GGG TGT TT
11βHSD-2 probe	CTC TGC GCC TCT CCA CTG TTT CAT GA
GR forward	GCG ATG GTC TCA GAA ACC AAA C
GR reverse	GCA GAG GAT AAC TTC CTC TGT AAT CTC
GR probe	TCA GAG CCT CAG CAA CCT TCA CTG CA
MR primer/probes from Assay on Demand; reference Hs00230908_m1. Sequences unavailable but pre-validated by ABI.	

Analysis of data was carried out by one-way analysis of variance, as described in section 2.5.

3.2.4 Immunohistochemistry

11βHSD-1, 11βHSD-2 and GR immunohistochemistry was performed by standard methods as described in 2.6 using biotin-conjugated secondary antibodies and ABC-Elite avidin biotin peroxidase complex (Vector, Milton Keynes, UK). Immunoreactivity was detected using the chromagen, 3,3'-diaminobenzidine (DAB). Semi-quantitative visual analysis and statistics were performed as described in 2.6.10.

MR immunostaining was carried out using a BOND-X automatic immunostaining robot (Vision Biosystems), with "define polymer with counterstain" reagent kit as described in section 2.6. This automated immunostaining process involves a peroxidase block prior to application of primary antibody, biotinylated secondary antibody (consisting of goat anti-mouse IgM, horse anti-mouse IgG and horse anti-rabbit IgG), followed by streptavidin-HRP conjugate, 3,3-diaminobenzidine detection and haemotoxylin counterstain. Prior to loading into the immunostaining robot, sections were dewaxed in xylene, rehydrated through graded alcohols and antigen retrieval performed as described in 2.6.2.

The 11βHSD-1 antibody was a sheep polyclonal obtained from Dr Karen Chapman, Centre for Cardiovascular Science, University of Edinburgh. The antibody was

raised in sheep against recombinant mouse 11βHSD-1. Commercial primary antibodies were used against 11βHSD-2 (PC545, The Binding Site, Birmingham, UK) and GR (NCL-GCR, NovoCastra, Newcastle-upon-Tyne, UK). The MR monoclonal antibody (MRN2-2D6) was one of a series produced by Celso Gomez-Sanchez (University of Mississippi) by immunizing mice with a peptide corresponding to amino acids 64-82 of the rat mineralocorticoid receptor (SKEKHELLPYIQQDNSRSG-C) conjugated to keyhole limpet hemocyanin. Resulting lymphocytes were fused with SP-2-mIL6 myeloma cells. This antibody is an IgG2b isotype and cross-reacts with the human mineralocorticoid receptor. The conditions used for each antibody are shown in Table 3.5.

Table 3.5 Immunohistochemical conditions

Primary Antibody	Dilution	Secondary antibody	Antigen retrieval	Controls
11βHSD-1	1:1500	Rabbit anti-sheep	No antigen retrieval	+ve: human liver; -ve matched IgG
11βHSD-2	1:4000	Rabbit anti-sheep	Pressure cooking, 0.01M NaCitrate pH6, 5 mins	+ve: human kidney; -ve matched IgG
GR	1:40	Horse anti-mouse	Pressure cooking, 0.01M NaCitrate pH6, 5 mins	-ve: matched IgG
MR	1:50	BOND-X secondary (goat anti-mouse IgM, horse anti-mouse IgG and horse anti-rabbit IgG)	Pressure cooking, 0.01M NaCitrate pH6, 5 mins	+ve: human kidney; -ve: pre-absorbed primary

Note: Performing antigen retrieval had no effect on the pattern of staining but increased the intensity. In the case of 11βHSD-1, some background staining occurred when antigen retrieval was performed.

3.2.5 Western Blotting

Western blotting was carried out to determine specificity of previously untested antibodies (against 11βHSD-1 and MR). The procedure was carried out using the standard technique described in 2.5. Antibody dilutions and control tissues are listed in Table 3.6.

Table 3.6 Western Blotting Conditions

Primary Antibody	Species	Dilution	Secondary antibody & dilution	Control tissue
MR	Mouse	1:100	Anti-mouse HRP-linked, 1:5000	placenta
11βHSD-1	Sheep	1:10,000	Anti-sheep HRP linked, 1:5000	liver

3.3 Results

3.3.1 Confirmation of Antibody Specificity

Specificity of the 11βHSD-1 and MR antibodies was confirmed using western blotting as they had not been previously validated. Both antibodies showed a clear, single band at the appropriate molecular weight in control tissues. Pre-adsorption with the appropriate peptide as these peptides were not available. Figure 3.1 shows 11βHSD-1 and Figure 3.2 shows MR on western blots.

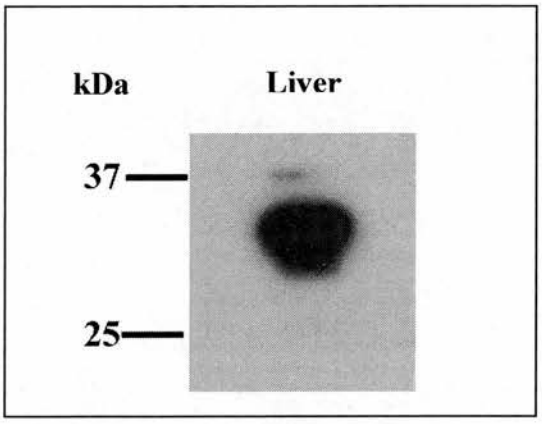


Figure 3.1 Western immunoblot of 11βHSD-1 in human liver, molecular weight 33kDa, as shown by western blotting.

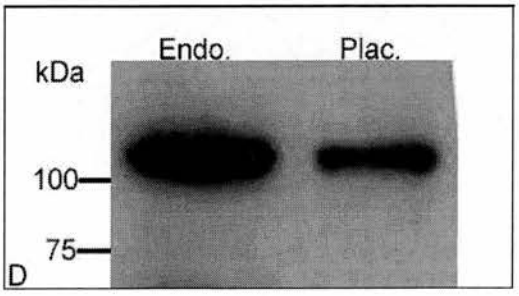
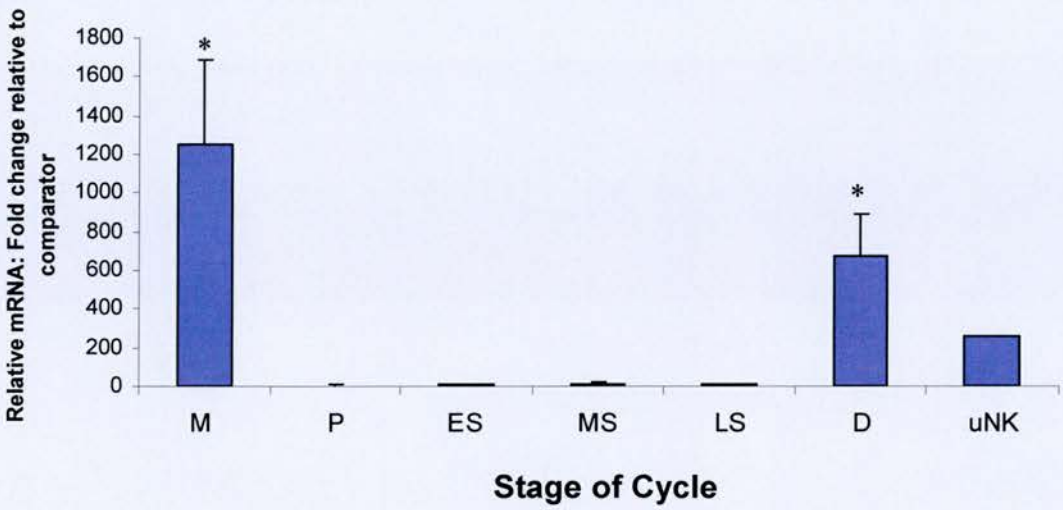


Figure 3.2 Western immunoblot of MR in human endometrium and placenta, molecular weight 102kDa, as shown by western blotting

3.3.2 Expression of 11βHSD-1mRNA in human endometrium

Levels of 11βHSD-1 mRNA were measured and validated using specific Taqman primers and probes as described. There were very low levels of 11βHSD-1 mRNA in normal endometrium, and this did not differ significantly between proliferative and secretory phases. The levels of 11βHSD-1 mRNA in menstrual endometrium were significantly increased in comparison to the rest of the menstrual cycle ($p<0.05$). Levels of mRNA were also significantly increased in first trimester decidua ($P<0.05$). There was also some increase in 11βHSD-1 mRNA levels in uterine natural killer (uNK) cells, a group of phenotypically unique cells that increase in number prior to pregnancy, in comparison to non-pregnant endometrium. These data are shown in Figure 3.3.



n =	4	4	4	4	4	5	1

Figure 3.3 Expression of 11βHSD-1 mRNA across the menstrual cycle, in first-trimester decidua, and uNK cells, mean ± SEM. M = menstrual, P = proliferative, ES = early secretory, MS = mid secretory, LS = late secretory, D = decidua, uNK = uNK cells. The comparator was a proliferative endometrium sample. *P<0.05 compared to all other stages of the cycle.

3.3.3 Expression of 11βHSD-1 protein in human endometrium

11βHSD-1 protein was expressed at low levels throughout the menstrual cycle. Expression was localised to the glandular and surface epithelia, with very low expression seen in the endothelial and stromal cells. There was no significant difference in expression patterns between the functional and basal layers. Intensity of expression did not differ significantly across the menstrual cycle, but there was a trend towards increased expression in menstrual endometrium.

11βHSD-1 protein was localised to the same cell types in first trimester decidua as in non-pregnant endometrium, the glandular and surface epithelium. Expression levels of the protein were seen to be slightly greater in first trimester decidua than endometrium, however this was not statistically significant.

These data are shown in Figures 3.4 and 3.5. Charts showing immunoscores are included in Figure A1 in Appendix 1.

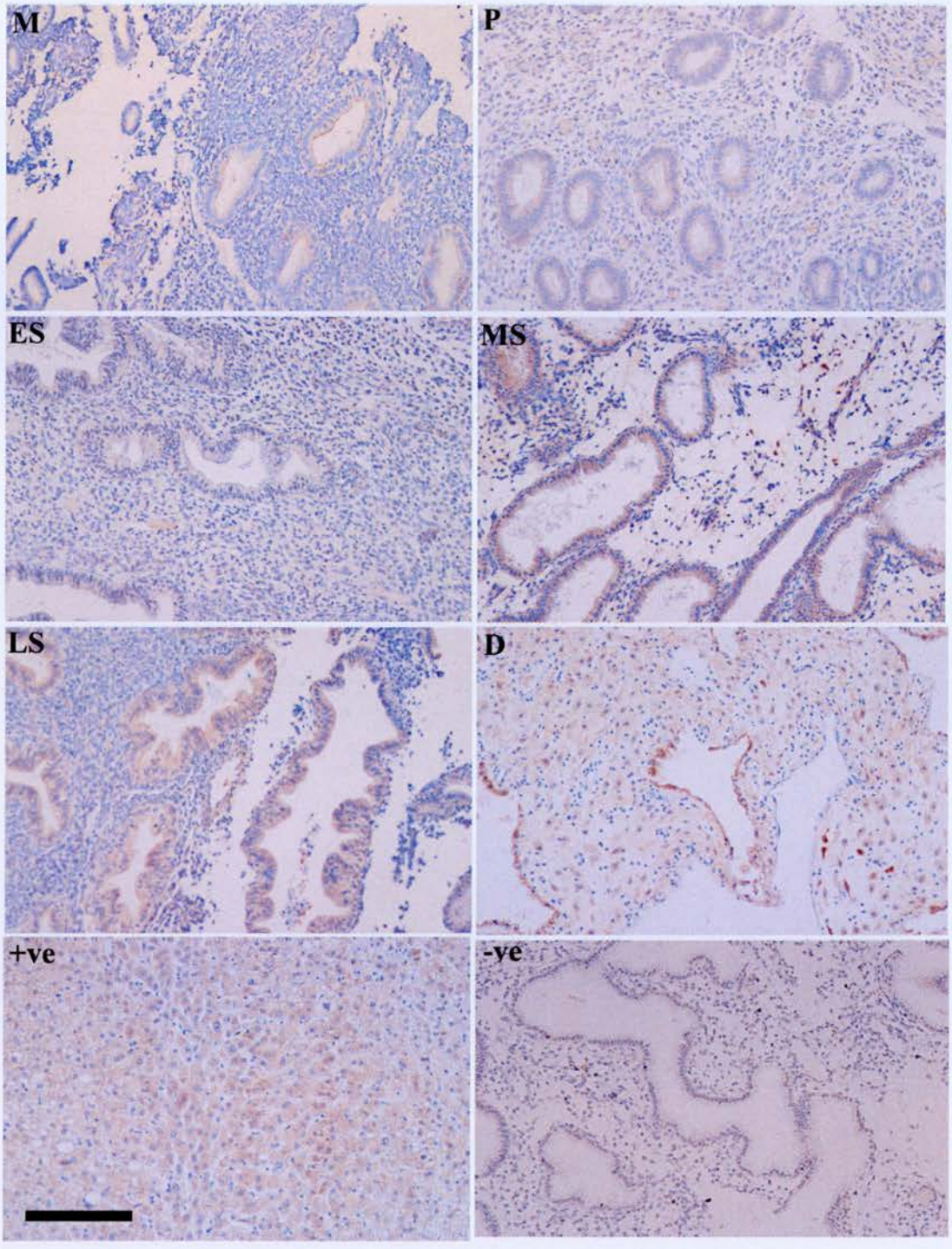


Figure 3.4 Immunolocalisation of 11βHSD-1 protein in the functional layer of the endometrium and decidua. M=menstrual, P=proliferative, ES=early secretory, MS=mid secretory, LS=late secretory, D=decidua, +ve= positive control, human liver, -ve=negative control, primary antibody substituted for matched IgG. Scale bar=10 microns.

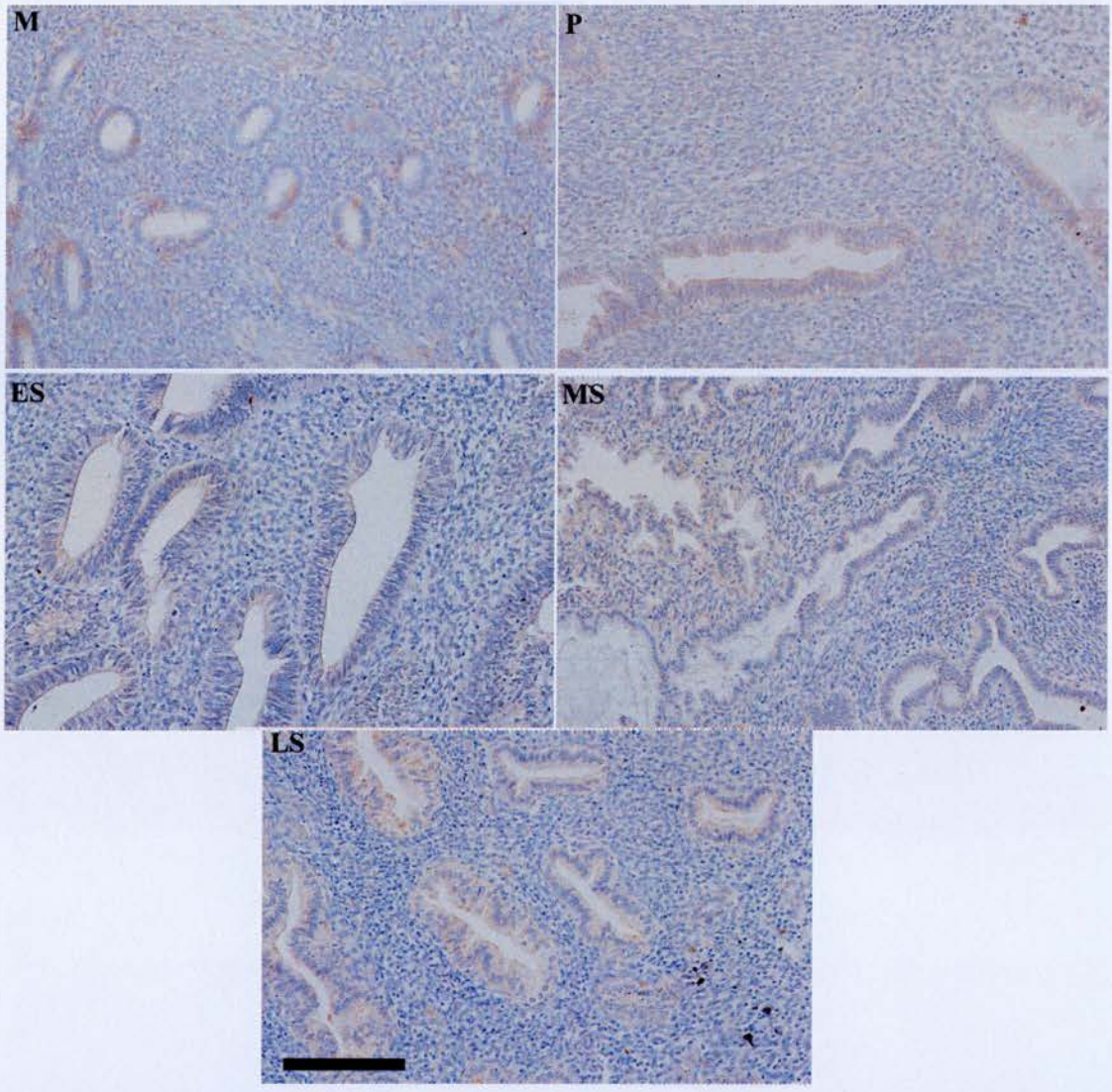


Figure 3.5 Immunohistochemical localisation of 11βHSD-1 in the basal layer of the endometrium. M=menstrual, P=proliferative, ES=early secretory, MS=mid secretory, LS=late secretory. Scale bar=10 microns.

3.3.4 Expression of 11βHSD-2 mRNA in human endometrium

11βHSD-2 mRNA was moderately expressed in normal endometrium throughout the menstrual cycle. There was no increase in 11βHSD-2 mRNA levels in menstrual phase endometrium. Levels of 11βHSD-2 mRNA in first trimester decidua did not differ significantly from those in normal endometrium. 11βHSD-2 mRNA was not detectable in uNK cells. These data are shown in Figure 3.6

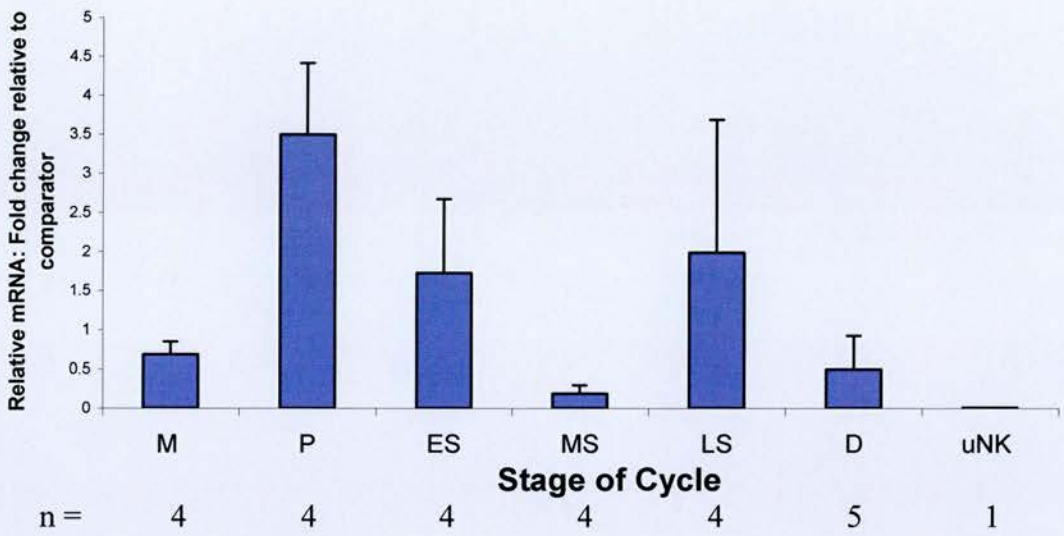


Figure 3.6 Expression of 11βHSD-2 mRNA across the menstrual cycle, in first-trimester decidua, and uNK cells, mean ± SEM. M = menstrual, P = proliferative, ES = early secretory, MS = mid secretory, LS = late secretory, D = decidua, uNK = uNKr cells. The comparator was proliferative endometrium.

3.3.5 Expression of 11βHSD-2 protein in human endometrium

11βHSD-2 protein expression and localisation was studied across the menstrual cycle and in first trimester decidua. 11βHSD-2 protein was expressed in the cytoplasm. 11βHSD-2 protein was localised in the glandular epithelium in both the functional and basal layer of the endometrium across the cycle, and at a greater level in first trimester decidua. Low levels of the protein were seen in the stroma of the functional layer, with an increase in first trimester decidua compared to normal endometrium. 11βHSD-2 protein was not present in the stroma of the basal layer. There was negligible expression in the endothelial cells of blood vessels of the both layers across the cycle, but some expression was seen in these cells in the functional layer of first trimester decidua. There was moderate expression of 11βHSD-2 protein in the surface epithelium of normal endometrium, and higher levels in first trimester decidua. Expression did not vary significantly in any part of the endometrium across the normal menstrual cycle. Figures 3.7 and 3.8 illustrate 11βHSD-2 immunoreactivity studies. Charts showing immunoscores are included in Figure A2 of Appendix 1.

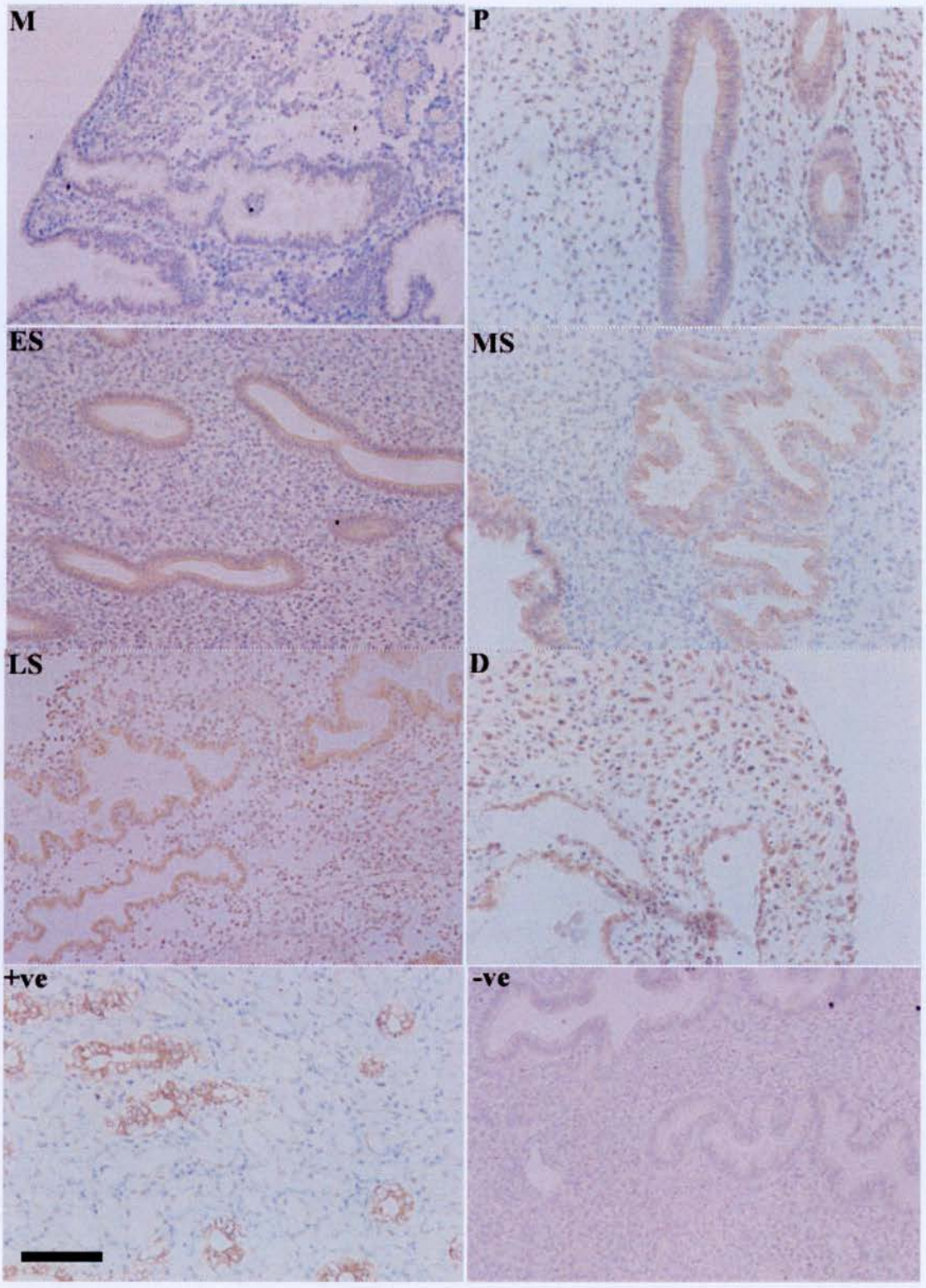


Figure 3.7 Immunolocalisation of 11βHSD-2 protein in the functional layer of the endometrium and decidua. M=menstrual, P=proliferative, ES=early secretory, MS=mid secretory, LS=late secretory, D=decidua, +ve= positive control, human kidney, -ve=negative control, primary antibody substituted for matched IgG. Scale bar= 10 microns.

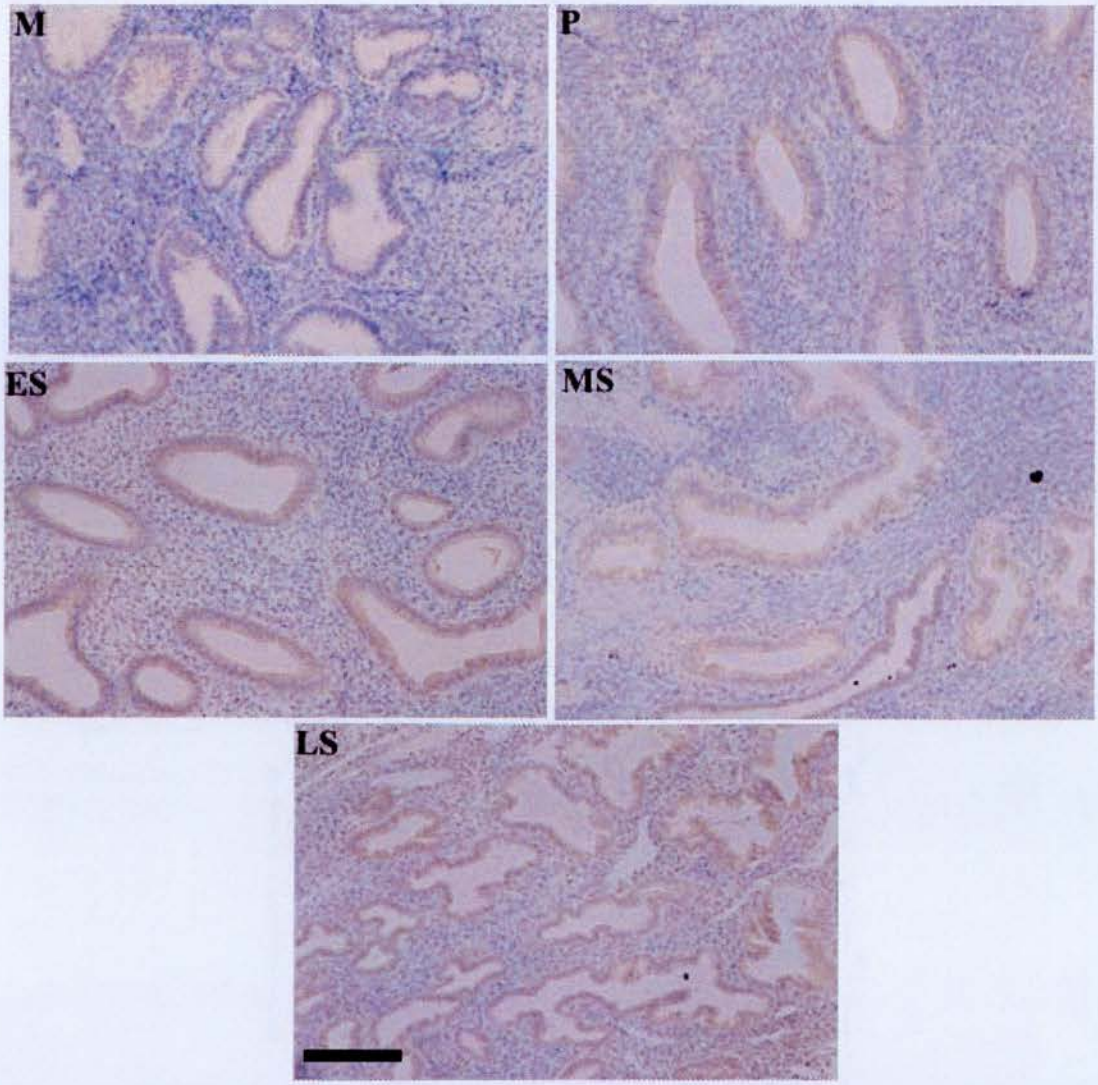


Figure 3.8 Immunohistochemical localisation of 11βHSD-2 in the basal layer of the endometrium. M=menstrual, P=proliferative, ES=early secretory, MS=mid secretory, LS=late secretory.Scale bar=10 microns.

3.3.6 Expression of GR mRNA in human endometrium

Expression of GR mRNA was also assessed by Taqman QRT-PCR. It was found that the mRNA was present in normal endometrium at low levels across the cycle and in first trimester decidua, with no significant difference in expression between stages of the cycle. However, expression levels were higher in menstrual endometrium. Figure 3.9 illustrates the expression pattern of GR immunoreactivity in endometrium across the menstrual cycle and decidua.

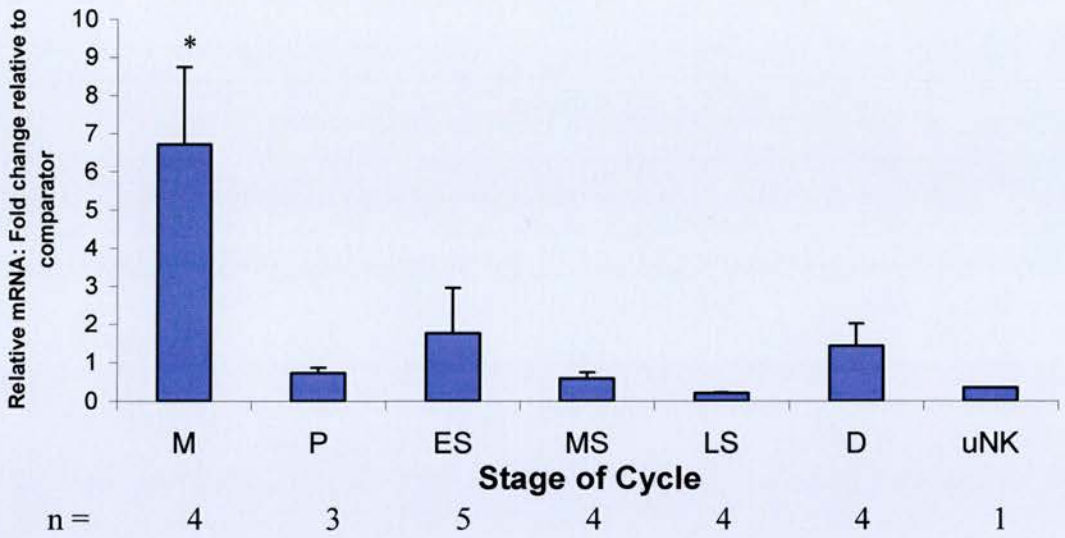


Fig. 3.9 Expression of GR mRNA across the menstrual cycle, in first-trimester decidua, and uNK cells, mean ± SEM. M = menstrual, P = proliferative, ES = early secretory, MS = mid secretory, LS = late secretory, D = decidua, uNK = uNK cells. The comparator was a proliferative endometrium sample. *P<0.05 compared to all other stages of the cycle.

3.3.7 Expression of the glucocorticoid receptor (GR) protein in human endometrium

GR protein was expressed in the nuclei of stromal cells at fairly high levels. There was no significant difference in expression across the cycle or in first trimester decidua. Expression of GR was low in glandular epithelium in both the functional and basal layers across the cycle. There was a significant increase in expression in decidual glands ($p<0.001$ by Kruskall-Wallis test as described in 2.6.11). There was high expression of GR in the endothelial cells of the vasculature in both functional and basal layers of the endometrium, and in first trimester decidua, with no significant difference across the cycle. Expression of GR protein increased across the cycle in the surface epithelium, from negligible expression in the proliferative and early secretory phases to weak expression in the late secretory phase. There was high expression of the protein in all cell types of first trimester decidua. Figures 3.10 and 3.11 illustrate patterns of immunoreactivity in the functional layer of the endometrium. Charts showing immunoscores are included in Figure A3 of Appendix 1.

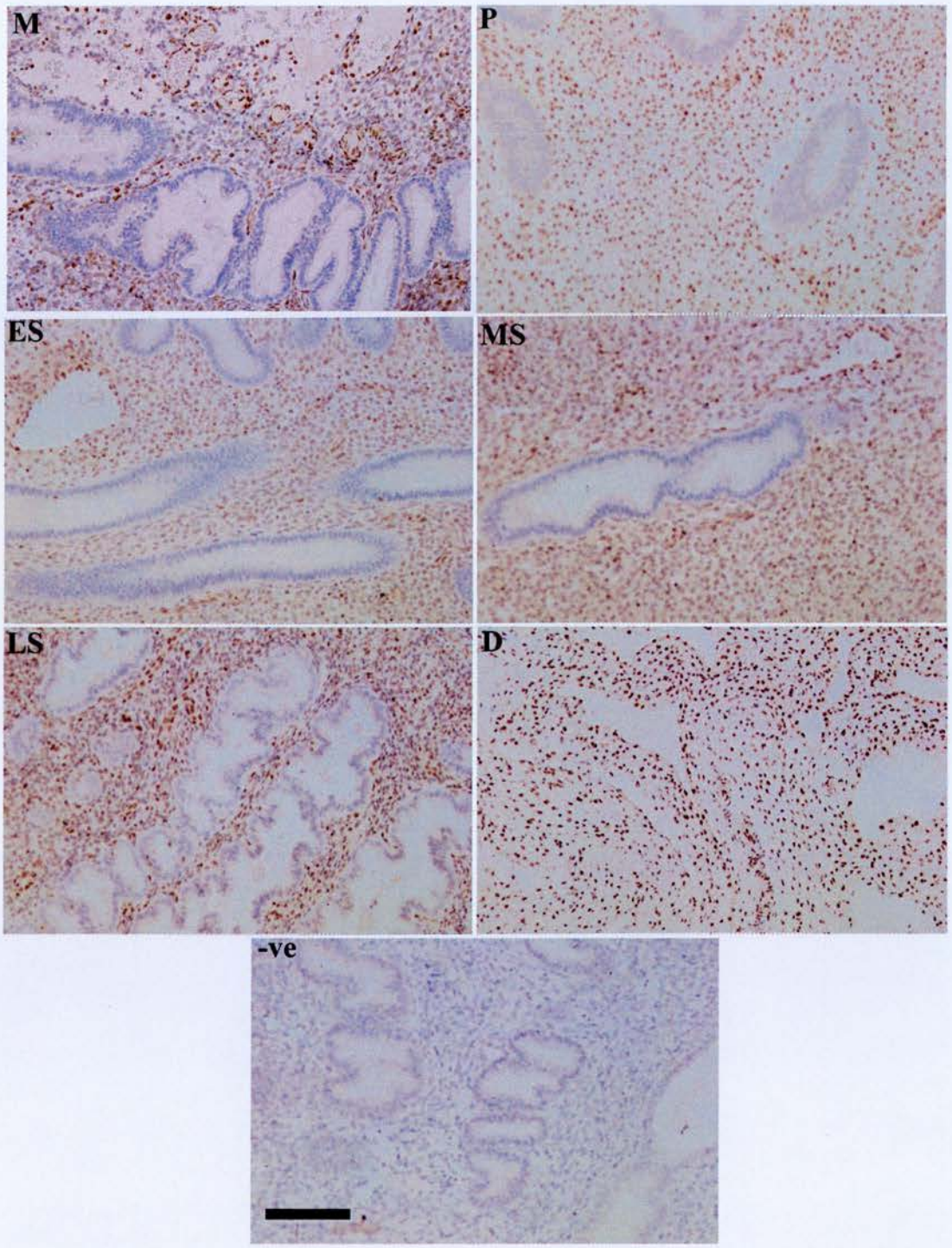


Figure 3.10 Immunolocalisation of GR protein in the functional layer of the endometrium and decidua. M=menstrual, P=proliferative, ES=early secretory, MS=mid secretory, LS=late secretory, D=deciduas, -ve=negative control, primary antibody substituted with matched IgG. Scale bar=10 microns.

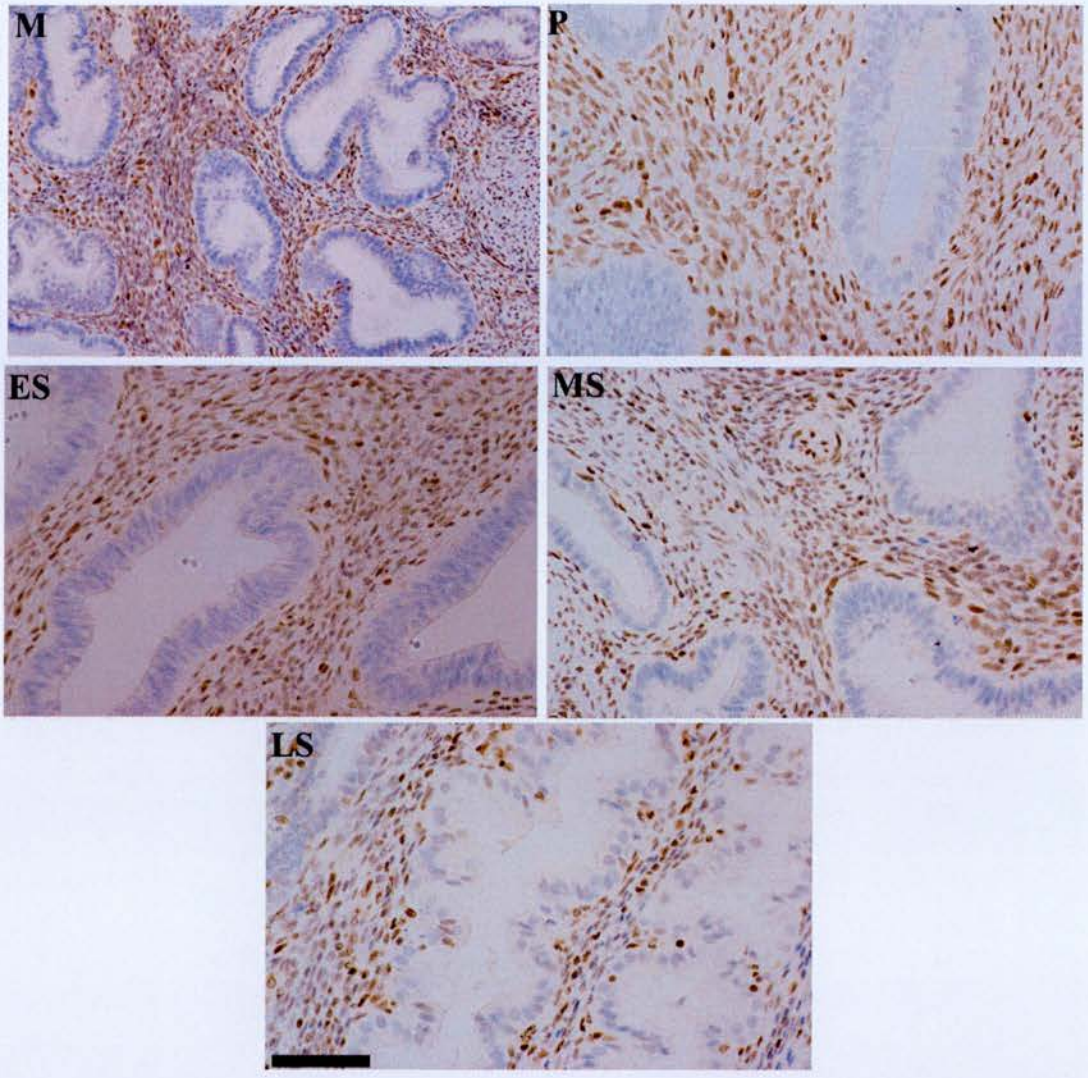


Figure 3.11 Immunohistochemical localisation of GR in the basal layer of the endometrium. M=menstrual, P=proliferative, ES=early secretory, MS=mid secretory, LS=late secretory. Scale bar=10 microns.

3.3.8 Expression of the mineralocorticoid receptor (MR) mRNA in human endometrium

MR mRNA was expressed throughout the menstrual cycle in non-pregnant endometrium. It was also expressed in first-trimester decidua and in an isolated population of uNK cells, although expression was very low in the latter. Expression levels are significantly higher in mid-secretory phase endometrium than in the proliferative phase. MR mRNA levels then fell significantly in the late secretory phase. There was an increase in the level of MR mRNA expression in first trimester decidua, but this result was not found to be statistically significant. These data are shown in Figure 3.12.

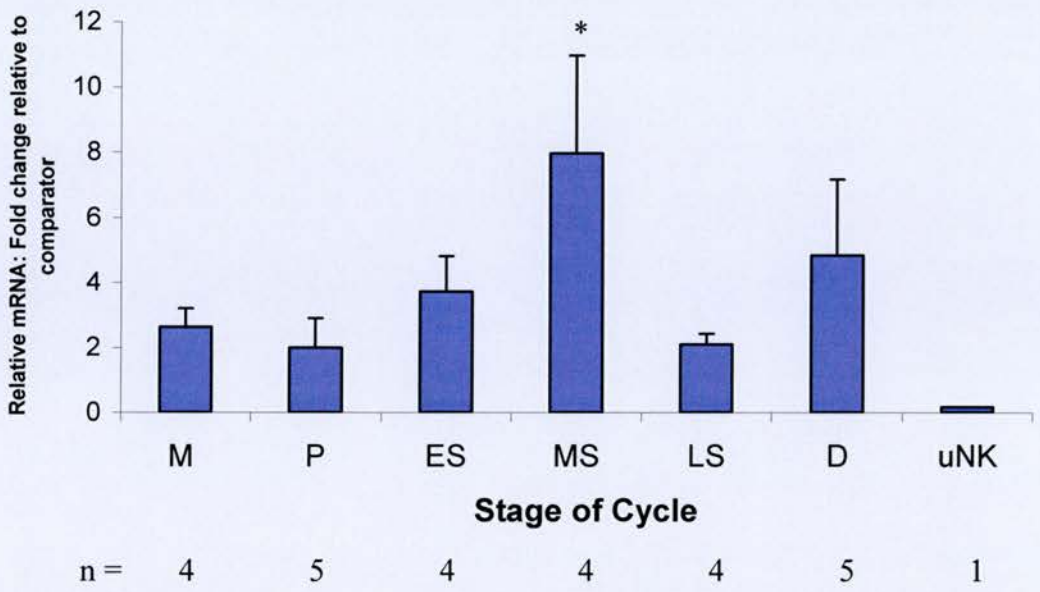


Fig. 3.12 Expression of MR mRNA across the menstrual cycle, in first-trimester decidua, and uNK cells, mean ± SEM. M = menstrual, P = proliferative, ES = early secretory, MS = mid secretory, LS = late secretory, D = decidua, uNK = uNK cells. The comparator was a proliferative endometrium sample. *P<0.05 compared to all other stages of the cycle.

3.3.9 Expression of the mineralocorticoid receptor (MR) protein in human endometrium

MR protein was found to be most strongly expressed in the glandular epithelia. There was variable, mild immunoreactivity seen in endometrial stromal cells and endothelial cells. Mild-moderate expression of MR protein was observed in the surface epithelium.

The expression pattern of MR protein in the basal layer of the endometrium was similar to that in the functional layer, although there was a trend towards reduced expression. There was very little expression of MR protein in the stromal cells or vasculature of the basal layer.

Expression of MR protein in first trimester decidua closely mirrored that of non-pregnant endometrium, with expression in the glandular epithelia, and some expression in stromal and endothelial cells. MR protein expression did not differ significantly between first trimester decidua and normal endometrium across the menstrual cycle.

MR protein was expressed variably in both the nucleus and cytoplasm in different tissues. In human kidney (positive control tissue) there was MR expression in the cytoplasm and punctate nuclear staining was also seen. In human endometrium there seemed to be predominantly cytoplasmic staining in the non-pregnant endometrium; however, nuclear staining was seen in first trimester decidua. These observations are

illustrated in Figures 3.13 and 3.14. Charts showing immunoscores are included in Figure A4 of Appendix 1.

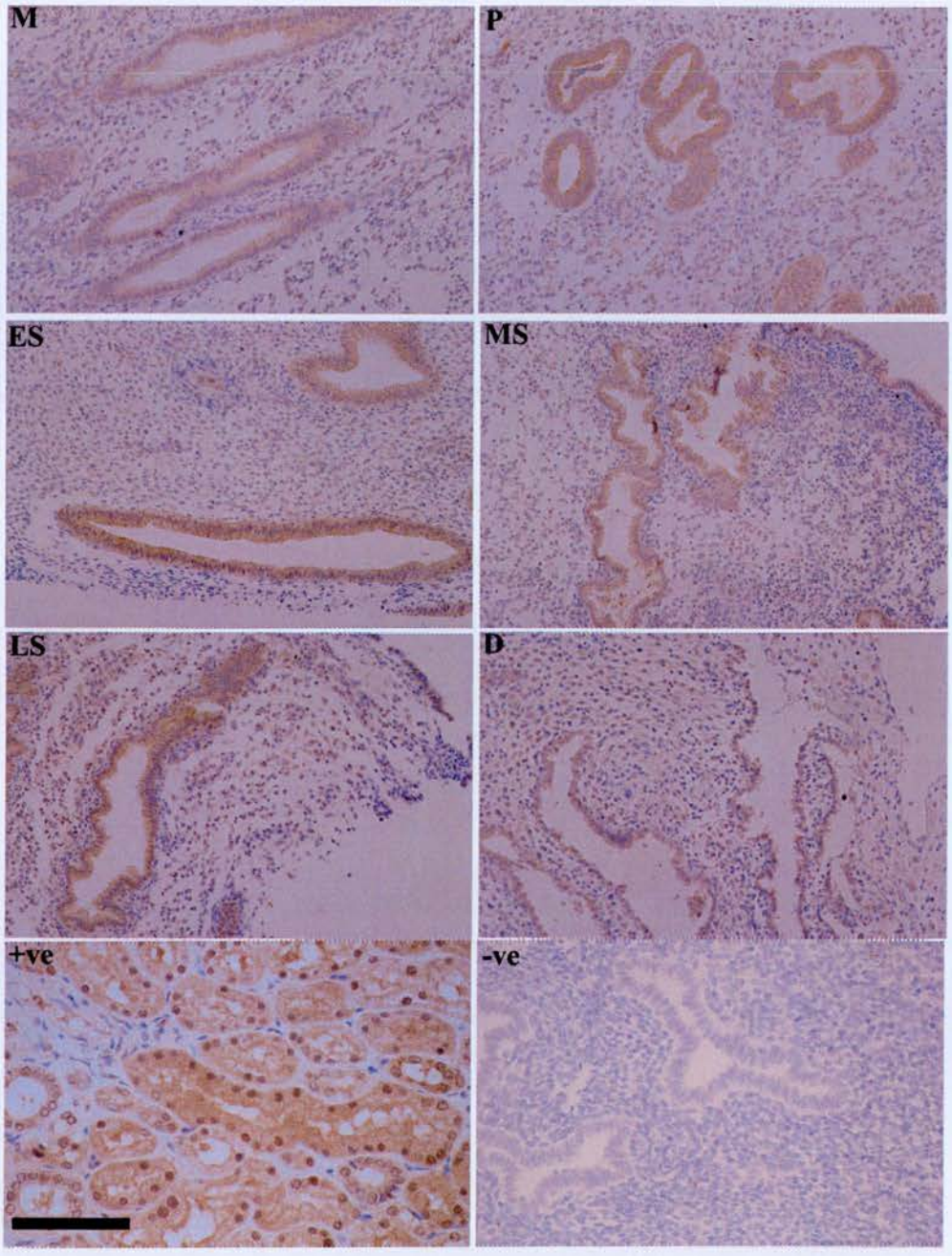


Figure 3.13 Immunolocalisation of MR protein in the functional layer of the endometrium and decidua. M=menstrual, P=proliferative, ES=early secretory, MS=mid secretory, LS=late secretory, D=decidua, +ve= positive control, human kidney, -ve=negative control, primary antibody preabsorbed against peptide. Scale bar=10 microns.

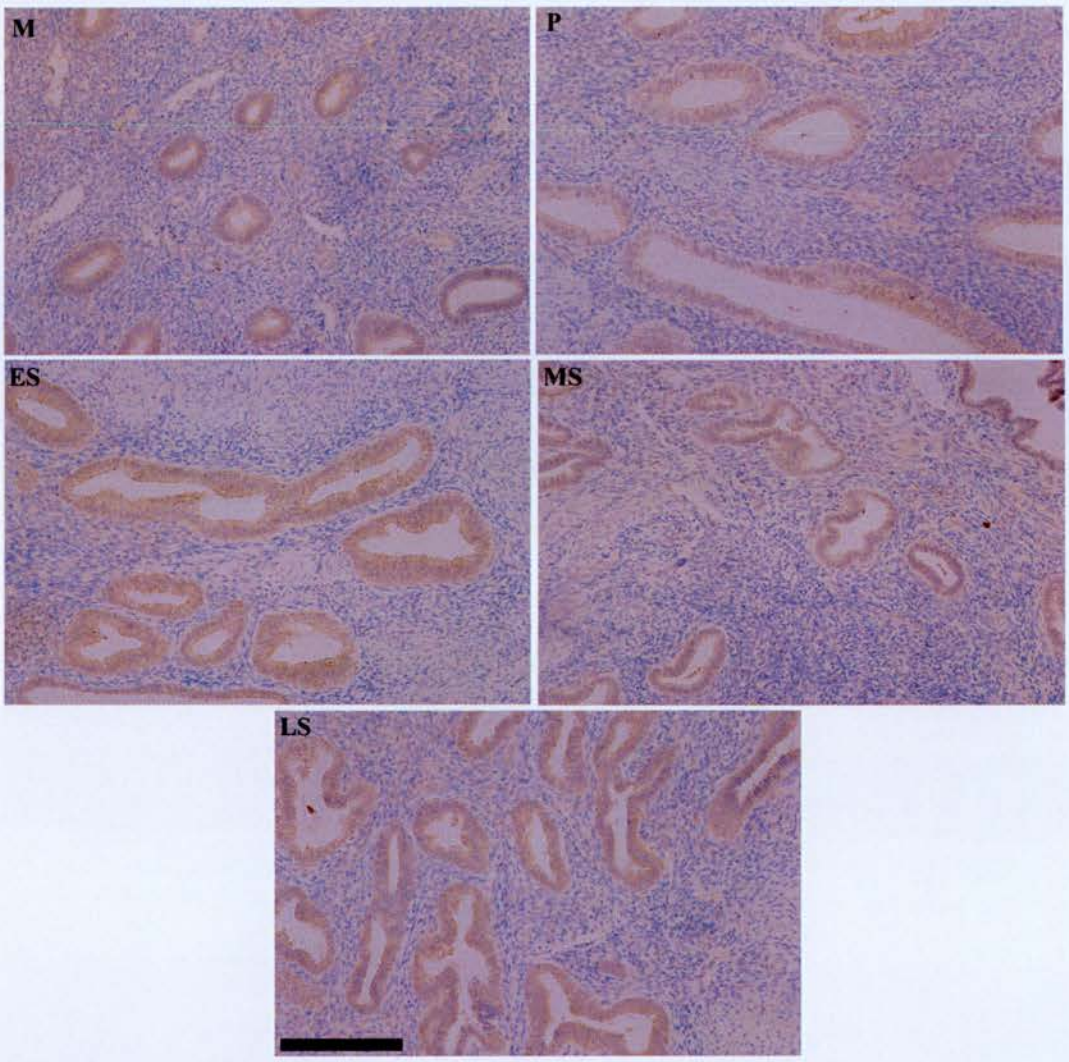


Figure 3.14 Immunohistochemical localisation of MR in the basal layer of the endometrium. M=menstrual, P=proliferative, ES=early secretory, MS=mid secretory, LS=late secretory. Scale bar=10 microns.

3.4 Discussion

These studies have shown for the first time the expression and localisation of 11βHSD-1 and MR protein in human endometrium across the menstrual cycle, and demonstrated the expression of 11βHSD-1 and -2, GR and MR mRNA and protein expression across the menstrual cycle in greater detail than has been previously reported. It has been shown that 11βHSD-1 expression is upregulated at menstruation and in first trimester decidua, whereas 11βHSD-2 expression does not vary across the cycle. GR is expressed throughout the menstrual cycle and levels of mRNA are increased at menstruation. MR is also expressed throughout the menstrual cycle and mRNA expression peaks in mid-secretory endometrium.

There was a large increase in 11βHSD-1 mRNA levels in endometrium at the time of menstruation in comparison to endometrium at other stages of the menstrual cycle. This may reflect an anti-inflammatory response, as both decidualisation and menstruation involve extensive remodelling of tissue. Correspondingly, levels of GR mRNA are also increased at menstruation.

MMPs are enzymes involved in the degradation of extracellular matrix proteins. Given the degree of injury and repair involved in menstruation, MMPs are likely to play a role. There may also be a link between 11βHSD-1 activity and MMP action, as proposed by Arcuri et al. (1996). It has also been shown that MMP levels are modulated by activation of the hypothalamic-pituitary-adrenal axis, of which cortisol is a product (Yang et al., 2001). Glucocorticoids are potent regulators of extracellular matrix degrading enzymes, of which MMPs are a large group.

11βHSD-1 mRNA levels were also greatly increased in uNK cells. These are a phenotypically unique cell type that increase in number with increasing circulating levels of progesterone in the non-pregnant secretory phase. The uNK cell population further increases in number in the first trimester of pregnancy. The increase in 11βHSD-1 mRNA expression in first trimester decidua may be a reflection of increased uNK cell numbers, a major component of the leukocyte population in first trimester decidua.

11βHSD-1 protein has been shown to be predominantly localised to the glandular epithelium of the endometrium and first trimester decidua. Expression is greatest in first trimester decidua, with a trend towards increased expression in menstrual endometrium. This pattern of protein expression is consistent with the mRNA data shown here, and lends further support to the suggestion that 11βHSD-1 plays a vital role in the injury and repair of the human endometrium at the time of menses. This also implies that the processes of implantation and menstruation are indeed of an inflammatory nature as suggested by Finn (1986). This is the first time 11βHSD-1 protein expression and localisation have been studied in such detail in human endometrium and it corroborates the novel aspects of the mRNA data shown here, namely the dramatic upregulation of 11βHSD-1 expression at menstruation and in first trimester decidua.

11βHSD-2 mRNA was expressed in human endometrium at all stages of the menstrual cycle and in first trimester decidua; expression did not differ significantly across the menstrual cycle.

It has been shown here that 11βHSD-2 protein is expressed in the cytoplasm of the glands in both the functional and basal layers of normal human endometrium, and also at low levels in the stroma of the functional layer. The level of expression remains constant across the menstrual cycle. Levels of 11βHSD-2 protein are increased in first trimester decidua, suggestive that 11βHSD-2 may well be involved in trophoblast invasion at the onset of pregnancy, by preventing cortisol-mediated inhibition of MMPs. Alternatively, the increase of 11βHSD-2 expression in first trimester decidua may be in balance with the dramatic upregulation of 11βHSD-1 mRNA.This may perhaps be to deliver protection to the fetus from excess circulating maternal glucocorticoids, which could be harmful. This response would not be crucial in the absence of pregnancy, thus there is no corresponding increase of 11βHSD-2 at menstruation.

11βHSDs may also be involved in the local regulation of various cellular events, by modulating glucocorticoid availability in a site-specific manner, including proliferation (Bigsby and Young, 1993), apoptosis (Terada et al., 1991; Jo et al., 1993), and biosynthesis of hormones, growth factors and enzymes such as MMPs (Makrigiannakis et al., 1992; Salamonsen, 1994). Hoffman et al. (1984) also suggested glucocorticoids may be involved in inhibition of implantation. This could explain the increase in 11βHSD-2 seen in first trimester decidua, to increase the synthesis of cortisone and thus decrease local cortisol levels, removing the local inhibitory effect.

GR mRNA was expressed in endometrium across the menstrual cycle, and mRNA levels did not differ significantly between the proliferative and secretory phases. This confirms data previously published by Bamberger et al. (2001) and Henderson et al. (2003). In menstrual endometrium, however, GR mRNA was upregulated compared to the rest of the menstrual cycle. This corresponds to the increase in expression of the enzyme that makes active ligand available (11βHSD-1), further suggesting a role for local glucocorticoid action in the inflammatory process of menstruation.

GR protein was expressed in endometrial stromal cells across the menstrual cycle and in decidualised endometrium. Levels of GR protein in the endometrial glands were up-regulated in first trimester decidua. This confirms data previously published (Henderson et al., 2003). GR is predicted to be co-localised with 11βHSD-1, and in the endometrium this is indeed the case in terms of temporal expression. However, GR is expressed only in the stroma of human endometrium, whereas 11βHSD-1 is largely confined to the glands. This suggests that the cortisol made available by 11βHSD-1 must be transported to the stroma in order to act on GR in a paracrine signalling manner. In first trimester decidua however, GR protein was also expressed in the glandular epithelia, the same location as the 11βHSD-1 enzyme. This would allow intracrine signalling to occur, which may elicit a faster glucocorticoid response than the paracrine signalling in non-pregnant endometrium.

A number of studies have shown that increased cortisol levels can lead to downregulation of GR in a number of cell types, including myoblasts (Whorwood et al., 2001), HeLa cells (Cidlowski et al., 1981; Shimojo et al., 1999), AtT-20 cells

(Svec et al., 1981) and COS-1 cells transfected with human GR (Burnstein et al., 1990). The studies in this thesis show an increase in expression of endometrial GR at the time cortisol levels would be expected to be greatest (due to increased 11βHSD-1 expression) – the menstrual phase. This may, however, be due to signals in the late secretory phase if pregnancy is not detected, to increase GR transcription. This increase would then be observed in the following stage of the cycle. Correspondingly, GR levels fall in the proliferative phase, following exposure to the expected high cortisol levels of the menstrual phase.

Cortisol has a greater affinity for MR than for GR and can act as a potent mineralocortocoid when it binds to MR. Excess binding of cortisol to MR can cause a number of disorders, in particular apparent mineralocorticoid excess and hypertension (Wilson et al., 1995). In order to prevent this, 11βHSD-2 is normally co-localised with MR. MR mRNA studies have shown that this indeed seems to be the case in a number of tissues including kidney (Edwards et al., 1988), colon (Funder et al., 1988), eye (Suzuki et al., 2001) and placenta (Hirasawa et al., 2000). Immunohistochemical localisation has confirmed this by showing both 11βHSD-2 and MR to be predominantly expressed in endometrial epithelial cells.

There have also been reports of GR and MR, or GR alone being co-expressed with 11βHSD-2, in particular in fetal tissues (Condon et al., 1998). It has been proposed that during pregnancy the role of 11βHSD-2 is to protect the fetus from relative maternal hypercortisolaemia (Driver et al., 2001). This suggests that glucocorticoid excess is more harmful to the fetus than mineralocorticoid excess.

MR is expressed across the normal menstrual cycle and in first trimester decidua, with an increase at the time of increased post-ovulatory progesterone concentrations, and a decrease in expression when progesterone is withdrawn, suggesting MR is directly regulated by progesterone. It has also been demonstrated that progesterone is able to bind to MR, however, it cannot elicit a mineralocorticoid response (Rupprecht et al., 1993; Quinkler et al., 2003). Progesterone thus appears to be acting as an antagonist of MR function. McDonnell et al. (1994) proposed a mechanism whereby MR transcriptional activity is repressed by ligand-activated PR-A via cross-talk of MR with this potent transdominant inhibitor competing for a common transcriptional factor or adaptor.

In the excretory systems, this process happens across the epithelia lining the distal parts of these systems (Horisberger & Rossier, 1992). It is therefore sensible to propose a similar mechanism in the endometrial epithelia, and correspondingly MR was found to be primarily expressed in glandular and surface epithelia.

Immunohistochemical data herein shows that MR expression in human endometrium is primarily cytoplasmic; however in first trimester decidua expression is both cytoplasmic and nuclear. This corresponds with the pattern of 11βHSD-1 expression, regulating the availability of ligand to bind MR. When 11βHSD-1 levels and thus cortisol levels are low, MR expression is predominantly cytoplasmic. In first trimester decidua, when 11βHSD-1 levels (and thus cortisol levels) are elevated, MR is observed in nuclei also. This suggests that MR translocates to the nucleus when ligand is bound, as previously reported (Sartorato et al., 2004).

The main function of the mineralocorticoid response is to regulate the balance of water and salts in tissues, in particular the balance of sodium and potassium. This is achieved by adjusting the transport rate of these salts. This would be a particularly crucial factor in the human endometrium at the time of pregnancy in order for the fetus to grow and develop normally. This could explain the increase seen in MR expression in the mid-secretory phase – the time when the endometrium receives signals to prepare for implantation. There was also a lesser, non-significant increase in MR mRNA in first trimester decidua which could be explained in a similar manner.

Interestingly, mRNA studies in uterine natural killer cells have shown that these cells express 11βHSD-1 and GR. These uNK cells however have negligible 11βHSD-2 or MR expression. There is currently little known about the precise role of uNK cells; however, it appears that glucocorticoid function is required whereas mineralocorticoid action is not. As there is very little MR present, 11βHSD-2 is not required to protect from excess cortisol binding. Previous studies have shown 11βHSD-1 mRNA and protein to be expressed in murine splenic lymphocytes (Zhang et al., 2005), and in human monocytes upon differentiation to macrophages (Thierenger et al., 2001). 11βHSD-2 was not seen to be present in either cell type.

MR protein expression in human endometrium across the menstrual cycle has not previously been reported. Here it has been demonstrated that MR is expressed across the menstrual cycle, however there are no significant differences in expression during the key events in the endometrium, menstruation and implantation.

As uNK cells are immune cells that increase greatly in number in the late secretory phase of the menstrual cycle, and further in first trimester decidua, it is possible they play a role in the inflammatory processes in the human endometrium here. A number of factors have been identified in the uNK cell population that implicate them in menstrual breakdown and endometrial regeneration, such as vascular endothelial growth factors (VEGF) and angiopoietin-2 (Li et al., 2001; reviewed by Moffett-King, 2002).

In order to further determine the expression patterns and roles of 11βHSD-1 and -2 in the endometrium, activity assays could be carried out. These studies would allow an accurate measurement of both the dehydrogenase and reductase activities of 11βHSD-1 and -2 by assaying their ability to metabolise substrates in the presence of different cofactors. This would also allow a comparative measure of 11βHSD-1 and -2 in the endometrium and thus give an accurate picture of the fine balance between the two enzymes.

Concluding Remarks

These data show that glucocorticoid expression and action are likely to have a key role in human endometrium. 11βHSD-1 has been shown to be expressed at greatest levels at times of extensive tissue remodelling, ie menstruation and early pregnancy, thus we can infer that there is a role for local modulation of cortisol expression in the human endometrium. Figure 3.15 shows a schematic summary of the data presented in this chapter.

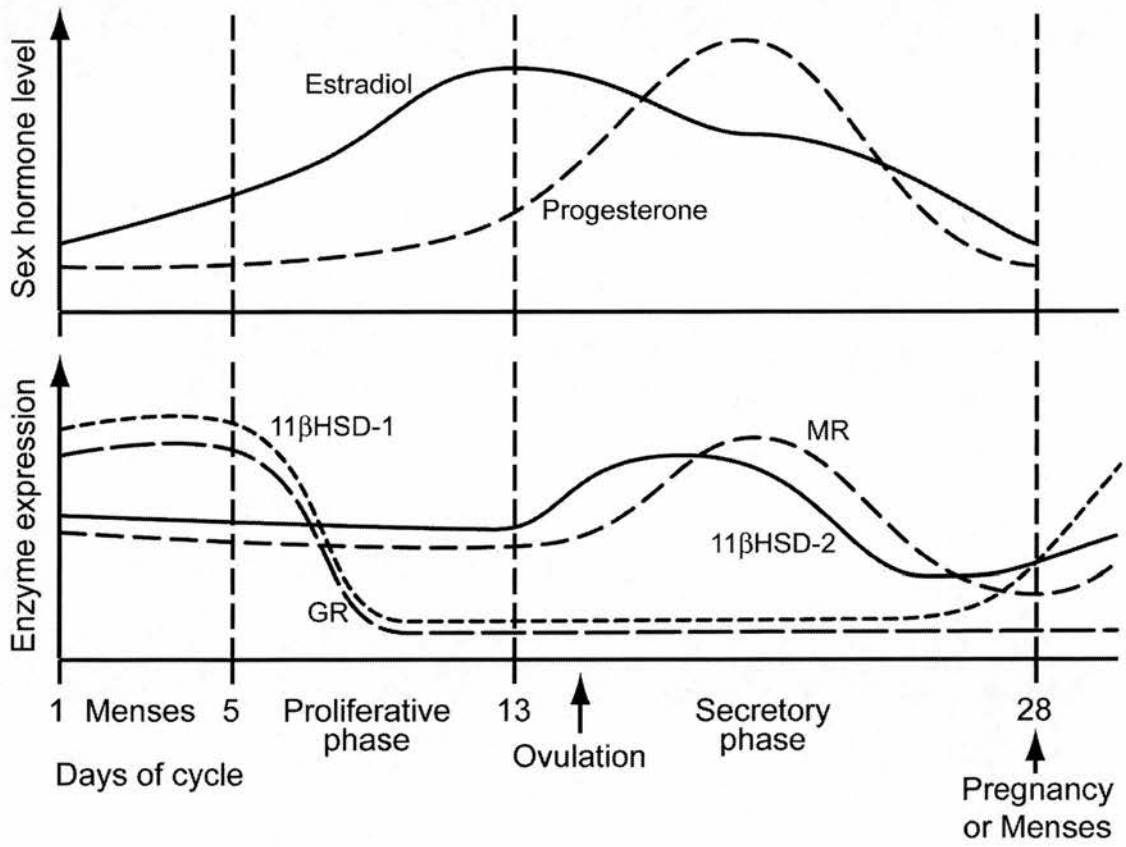


Figure 3.15 Schematic diagram summarising expression patterns of 11βHSD-1 and -2 enzymes, GR and MR across the menstrual cycle.

Chapter 4:

Cytokine control of glucocorticoid metabolism

4.1 Introduction

Cytokines are small glycoproteins that can have pro- and anti-inflammatory effects. They are produced by virtually all cells and can be split into two groups, T-helper 1 (TH1) and T-helper 2 (TH2) cytokines. TH1 cytokines generally have a pro-inflammatory action whereas TH2 cytokines are anti-inflammatory. Cytokines are expressed in the human endometrium by the lymphoid, stromal and epithelial cells (Hunt & Pollard, 1992; Tabibzadeh & Sun, 1992). A number of investigators have contributed to the hypothesis that a TH1 response is detrimental to pregnancy and TH2 response is beneficial (Wegmann et al., 1993; Lim et al., 1998; Hill & Choi, 2000).

11βHSD-1 and -2 act as a glucocorticoid "shuttle" interconverting the active and inactive glucocorticoids, cortisol and cortisone respectively in humans. Cortisol acts as an anti-inflammatory agent via the glucocorticoid receptor (GR), but also binds with high affinity to the mineralocorticoid receptor (MR). Consistent with cortisol's anti-inflammatory action, it is predicted that during periods of inflammation, 11βHSD-1 expression will predominate, as shown in section 3.3 of this thesis. The regulation and mechanism of action of 11βHSDs and the receptors for glucocorticoids, are consequently of interest.

Studies on the regulation of glucocorticoid-metabolising enzymes have shown that a number of factors including cytokines can modulate the expression of 11βHSD-1 and -2. Of these cytokines, a number of studies have focussed on the actions of

interleukins, in particular interleukin 1 (IL-1). This is a pro-inflammatory TH1 cytokine. There are two forms of IL-1, IL-1α and IL-1β. Both forms have similar action and act through the same receptor. IL-1β is not expressed in proliferative and early secretory endometrium; however, IL-1α is expressed throughout the cycle (Tabibzadeh & Sun, 1992). Studies in our laboratory have investigated the effects of IL-1α and a number of other cytokines on 11βHSD-1 mRNA expression. Rae et al. (2004a) and Yong et al. (2002) have found 11βHSD-1 to be upregulated by IL-1α in human ovarian surface epithelium cultures. They also found that the co-incubation with cortisol amplified this response.

A number of studies have shown a similar response in different cells for example glomerular mesangial cells (Escher et al., 1997) and cells of the airway (Feinstein & Schleimer, 1999). Thus it could be proposed that epithelial cells behave similarly regardless of the tissue type. Against this background, it would be expected that both the surface and glandular epithelia of the endometrium may behave in a similar manner to the ovarian surface epithelium. The endometrium, however, is a heterogeneous tissue, consisting of a large stromal component as well as epithelia and other cell types. The predominant cell type in the stromal compartment is a fibroblast-like cell. Thus, when investigating the effects of cytokines on glucocorticoid metabolism and action, it is desirable to study both stromal and epithelial cells.

The hypothesis is that endometrial epithelial cells are regulated in a similar manner to those in the ovarian surface epithelia, in that expression of 11βHSD-1 was increased in response to IL-1α and attenuated by cortisol.

Hence the aims of this study were:

To treat endometrial epithelial and stromal cells with increasing doses of IL-1α and measure response using the following end-points:

11βHSD-1 and -2, GR and MR mRNA expression.

4.2 Materials and Methods

4.2.1 Subjects and Tissue Collection

Ethical approval and informed consent was obtained from all women who agreed to participate in these studies. Endometrial biopsies were collected from 14 women attending the out-patient clinic for investigation of benign gynaecological complaints or for surgical management ie hysterectomy, as described in 2.1. Subjects were all healthy women of reproductive age, and taking no steroidal medication, however not all had regular menstrual cycles. Details of the samples are given in Table 4.1.

Table 4.1 Endometrium samples used in this chapter

Sample	E2 level at time of biopsy (pmol/l)	P4 level at time of biopsy (nmol/l)	Histological dating
1	59	1	Late secretory
2	504	72	Early secretory
3	479	20	Unclear
4	229	16.8	Proliferative
5	245	1.7	Unclear
6	150	3.1	Menstrual
7	266	58	Unclear
8	153	2.2	Inactive
9	356	118.9	Mid secretory
10	98	2.	Late secretory
11	479	4.2	Proliferative
12	856	18.5	Early secretory
13	255	24	Unclear
14	396	36.6	Mid secretory

4.2.2 Cell culture

Samples were collected in Dutch Modification RPMI culture medium (Sigma). The biopsies were then washed in Dulbecco's PBS (Sigma), and a cell separation protocol carried out as described in 2.8. Isolated stromal cells were then cultured in Dutch Modification RPMI medium (Sigma) containing fetal calf serum, penicillin, streptomycin, L-glutamine and gentamycin (all Sigma), known as "F" medium. Epithelially enriched cells and cells collected from the surface of the endometrium with a brush were cultured in "HOSE 1" medium as described in section 2.8. (1:1 vol/vol 199 medium:MCDB105, fetal calf serum, streptomycin, penicillin and L-glutamine; all Life Technologies). This collection and separation method is described in detail in 2.8.

4.2.3 Incubation of cells with IL-1α

When the cultured stromal, epithelially enriched and surface cells (as described in 2.8) were fully confluent, they were removed from culture flasks using 1x Trypsin/EDTA treatment (5 minutes at 37°C; reaction stopped with serum-containing medium), centrifuged at 2000rpm for 3 minutes and the supernatant discarded. The pellet was resuspended in the appropriate medium and cell counts performed on a small aliquot using a haematocytometer. Cells were diluted to a concentration of 200,000 cells/ml medium and transferred to 6-well 35mm plates (1ml/well). The volume in each well was adjusted to 3ml with serum-containing

medium. Cells were incubated overnight at 37°C, then transferred to serum-free medium. The cells were again cultured overnight at 37°C.

Cells were then treated with either 50, 500 or 1000pg/μl IL-1α in the presence or absence of 1μM cortisol in serum-free medium and cultured for 48 hours at 37°C.

All concentrations used were based on those used by Rae et al. (2004a) in their investigation of the effects of cytokines on glucocorticoid metabolism in ovarian surface epithelial cells. Where cortisol was added to the reaction mix, 10^{-3}M stock was added (usually 3μl) to give a final concentration of 1μM. Cells were treated with these reaction mixes at 37°C for 48 hours, then harvested. Cultures with no added cortisol had 3μl ethanol vehicle added instead.

4.2.4 Cell harvesting

Culture medium was removed from the cells. Two methods of removing cells from the culture plates were used. In the first method, 1% Trypsin/EDTA was applied as described in 4.2.3, then the cells were scraped with a pipette tip and transferred to a 2ml tube. These cell suspensions were centrifuged at 2000rpm for 5 minutes, supernatant removed and the pellet resuspended in lysis buffer from the Qiagen RNeasy mini-kit. Cells were then passed through a 19-gauge needle to break them and then frozen at -70°C for later RNA extraction.

After the initial experiments it was clear that the yield of RNA was not as great as would be expected, so an alternative method of cell harvesting was sought. This

involved washing the cells in PBS and pipetting lysis buffer directly onto the culture plates, before scraping the cells and repeatedly pipetting. The suspension was transferred to a 2ml tube, passed through a 19-guage needle and frozen at -70°C as before. A greater yield of RNA was obtained using this method.

4.2.5 Confirmation of cell type using cytokeratin staining

Epithelial cells were identified using immunocytochemistry with an antibody that detects cytokeratins 5, 6, 8 and 17 (Dako). This was done using standard immunohistochemical technique as described in 2.6, with the following modifications.

When cells were plated for culture, a small drop of each sample was placed in a well of a 6-well chamberslide (Nunc) and cultured at 37°C for 48 hours. Medium was then removed and the cells washed with PBS. Cells were then fixed for 10 minutes at -20°C in cold methanol and washed in PBS. Normal goat serum was then applied for 30 minutes and the primary antibody applied and incubated overnight at 4°C. The following day the cells were washed in PBS and the secondary antibody (biotinylated goat anti-mouse, Vector) applied for 30 minutes at room temperature. The cells were again washed in PBS and ABC-HRP solution applied (Dako) for 30 minutes. Following a wash with PBS, the plastic casing was removed and cells stained with DAB, counterstained with haemotoxylin and mounted in Pertex (Cellpath, Hemel Hempstead, UK).

4.2.6 RNA extraction and reverse transcriptase-PCR

RNA was extracted using an RNeasy Mini Kit (Qiagen) as described in 2.3.2, with the modification as described in the manufacturer's handbook and in 2.3.2 of this thesis. After freezing cell homogenates in lysis buffer, they were heated to 37°C in a water bath for 20 minutes before beginning the extraction with the addition of 70% ethanol. RNA was then stored at -70°C, and 1μl used to measure RNA concentration and quality using an Agilent 2000 Nano Bioanalyser as described in section 2.5. There was found to be no difference in RNA quality between the two cell harvesting methods.

Reverse transcriptase-PCR was performed using standard conditions as described in section 2.6.

4.2.7 Quantitative Real-Time PCR (QRT-PCR)

Oligonucleotide forward and reverse primers, and oligonucleotide Taqman probes were used to detect the sequences of interest. The probes used were designed using Primer Express software as previously described (11βHSD-1, -2, GR) (Rae et al., 2004a; Henderson et al., 2003) or commercially available from Applied Biosystems Assay on Demand service (MR). The sequences of the primer/probe sets have been shown previously in Table 3.4. Quantitative Real-time PCR was performed using a 7900 sequence detection system and analysed as described in section 2.7.

4.3 Results

4.3.1 Expression of 11βHSD-1 in response to treatment with IL-1α and cortisol

Expression of 11βHSD-1 was increased significantly in endometrial fibroblasts following treatment with IL-1α, however addition of cortisol had no effect. Maximal upregulation of 11βHSD-1 mRNA was seen with 500pg/ml IL-1α, with a 10-fold increase over untreated control. In an epithelially enriched population of cells, a similar trend was seen, with IL-1α causing an upregulation of 11βHSD-1 expression maximally at a dose of 500pg/ml, however addition of cortisol in this case showed a further increase. Cells taken from the surface of the endometrium exhibited a small increase in 11βHSD-1 expression with IL-1α treatment, although this increase did not become significant until the dose of IL-1α was 1000pg/ml. When 1μM cortisol was added however, a significant increase in expression of 11βHSD-1 was seen with only 50pg/ml IL-1α, and a highly significant increase at 500pg/ml. Cortisol alone had no effect. These data are shown in the charts in Figure 4.1.

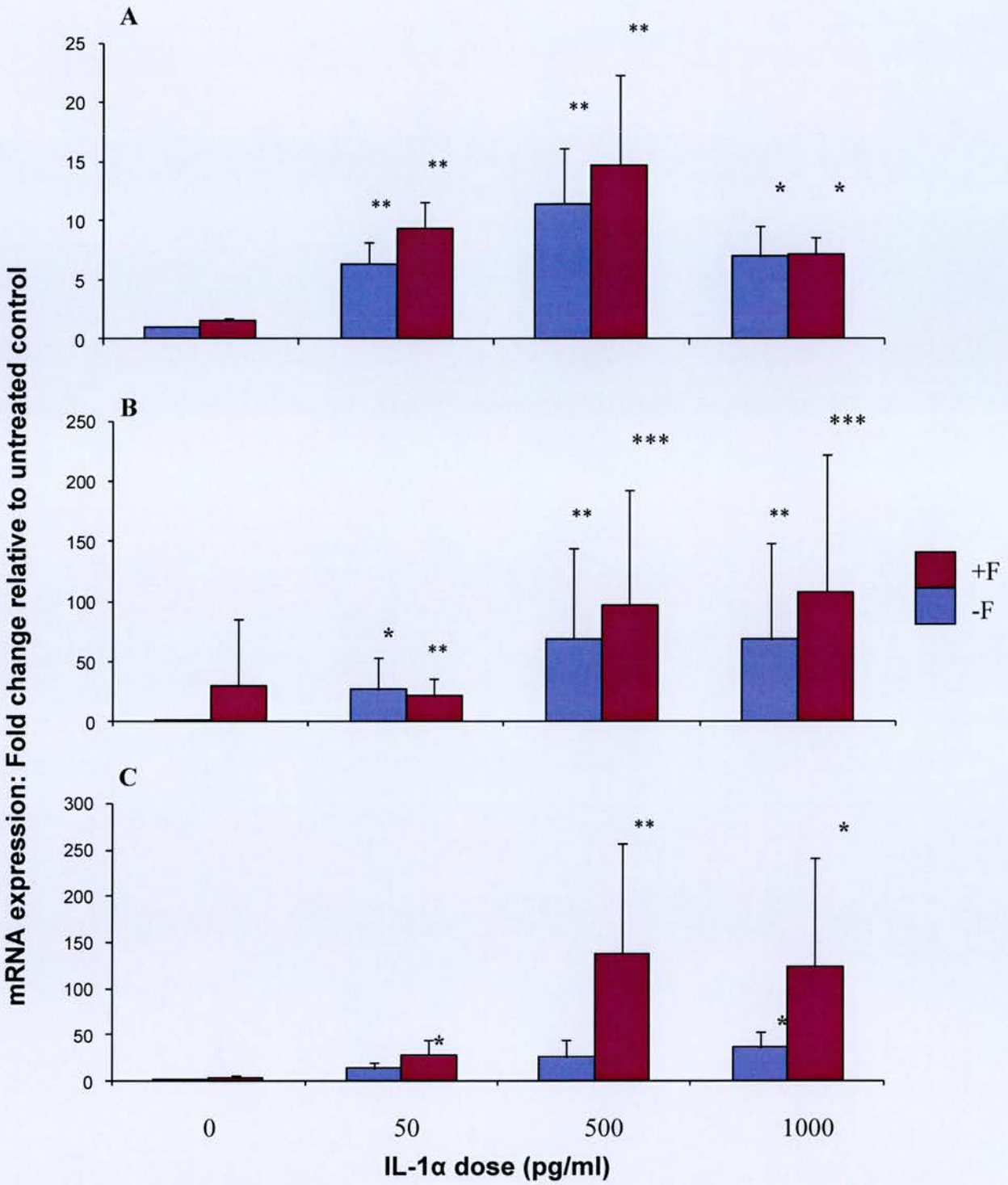


Figure 4.1 Expression of 11βHSD-1 mRNA in response to increasing doses of IL-1α in the presence and absence of cortisol, mean ± SEM. RNA levels are measured as a fold change relative to untreated control. A: endometrial stromal cells (n=5), B=epithelially enriched cells (n=4), C= mixed cells brushed from endometrial surface (n=5). The comparator was the untreated control for each sample.*p<0.05; **p<0.01, ***p<0.001 compared to untreated control. Note the differing scales on the vertical axis between the cell types. Stage of cycle had no significant bearing on response.

4.3.2 Expression of 11βHSD-2 in response to IL-1α and cortisol treatment.

No significant difference in 11βHSD-2 expression was seen in endometrial stromal cells when treated with increasing doses of IL-1α in the presence or absence of cortisol. In epithelially enriched cultures, there was a small increase in 11βHSD-2 expression with treatment with 50-500pg/ml IL-1α, however this was not statistically significant. Cortisol had no effect on 11βHSD-2 mRNA levels in these cells. 11βHSD-2 was not detectable by QRT-PCR in cells from the surface of the endometrium before or after treatment with IL-1α and/or cortisol. These data are shown in the charts in Figure 4.2.

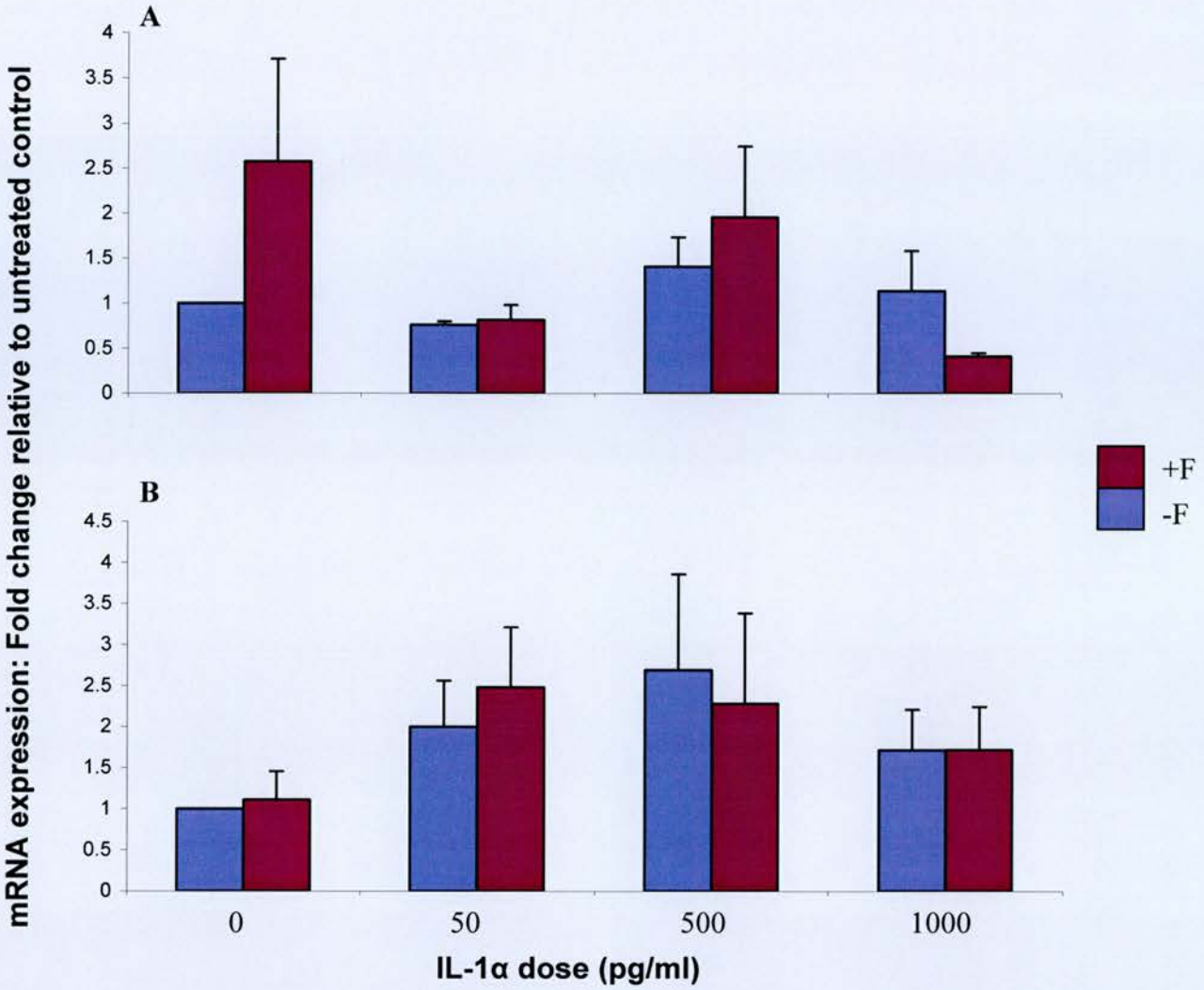


Figure 4.2 Expression of 11βHSD-2 mRNA in response to increasing doses of IL-1α in the presence and absence of cortisol, mean ± SEM. RNA levels are measured as a fold change relative to untreated control. A: endometrial stromal cells (n=5), B=epithelially enriched cells (n=4). The comparator was the untreated control for each sample. 11βHSD-2 was not detectable in cells from the endometrial surface.

4.3.3 Expression of GR in response to IL-1α and cortisol

GR mRNA was expressed in all cell types studied, but expression was not significantly changed in endometrial stromal cells in response to either IL-1α or cortisol treatment. In an epithelially enriched population of cells, there was a trend towards increasing GR mRNA expression after treatment with 50pg/ml IL-1α; however, this was not statistically significant. There was no further increase seen with increasing doses of IL-1α, or with the addition of cortisol. GR expression in cells from the surface of the endometrium did not change in response to IL-1α or cortisol treatment. These data are shown in Figure 4.3.

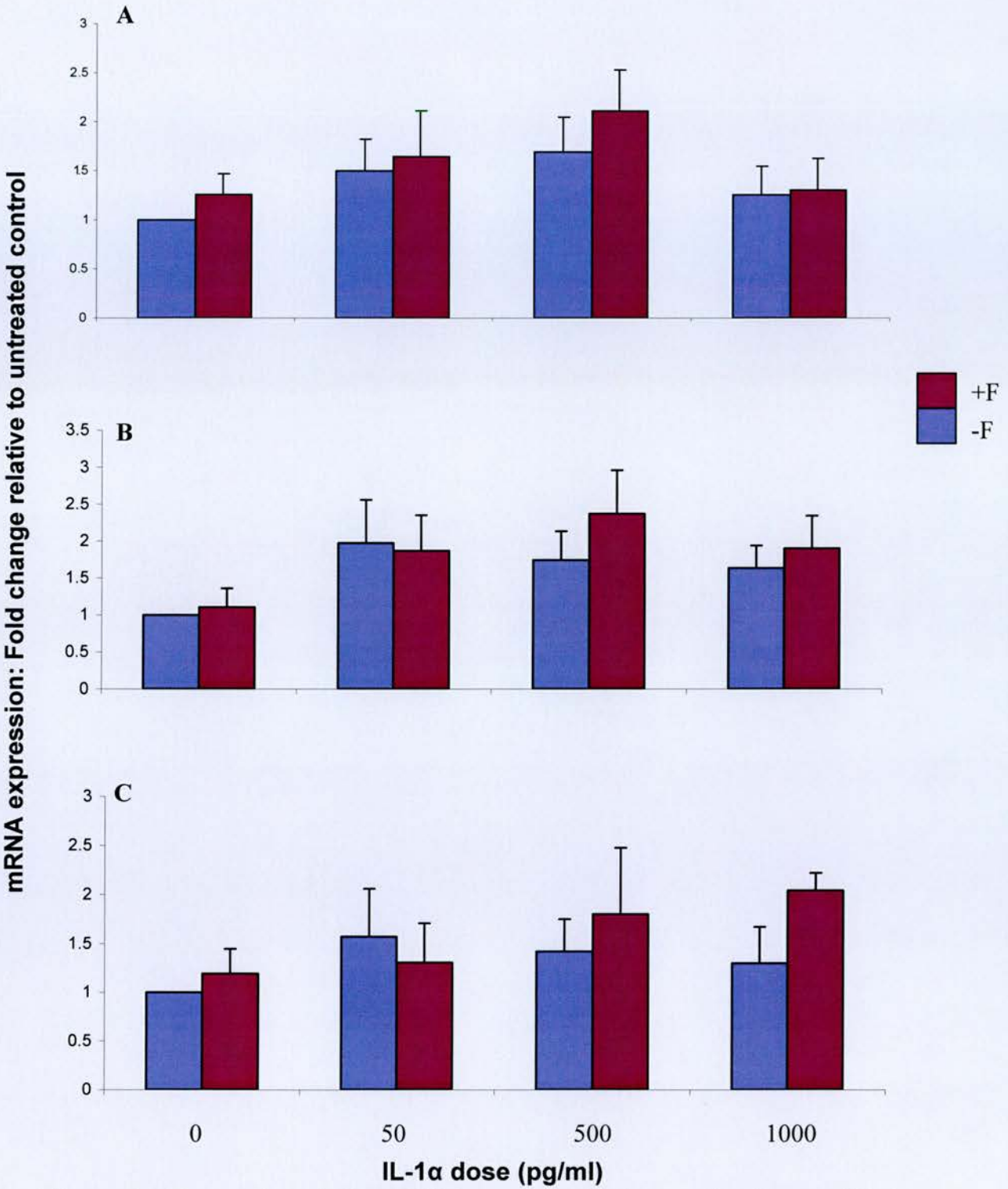


Figure 4.3 Expression of GR mRNA in response to increasing doses of IL-1α in the presence and absence of cortisol, mean ± SEM. RNA levels are measured as a fold change relative to untreated control. . A: endometrial stromal cells (n=5), B=epithelially enriched cells (n=4), C= mixed cells brushed from endometrial surface (n=5). The comparator was the untreated control for each sample.

4.4.4 Expression of MR in response to IL-1α and cortisol.

There was no significant change in expression of MR mRNA in response to IL-1α or cortisol treatment in either endometrial stromal, epithelial, or surface cells. MR mRNA was expressed in all 3 cell populations however. These data are shown in Figure 4.4.

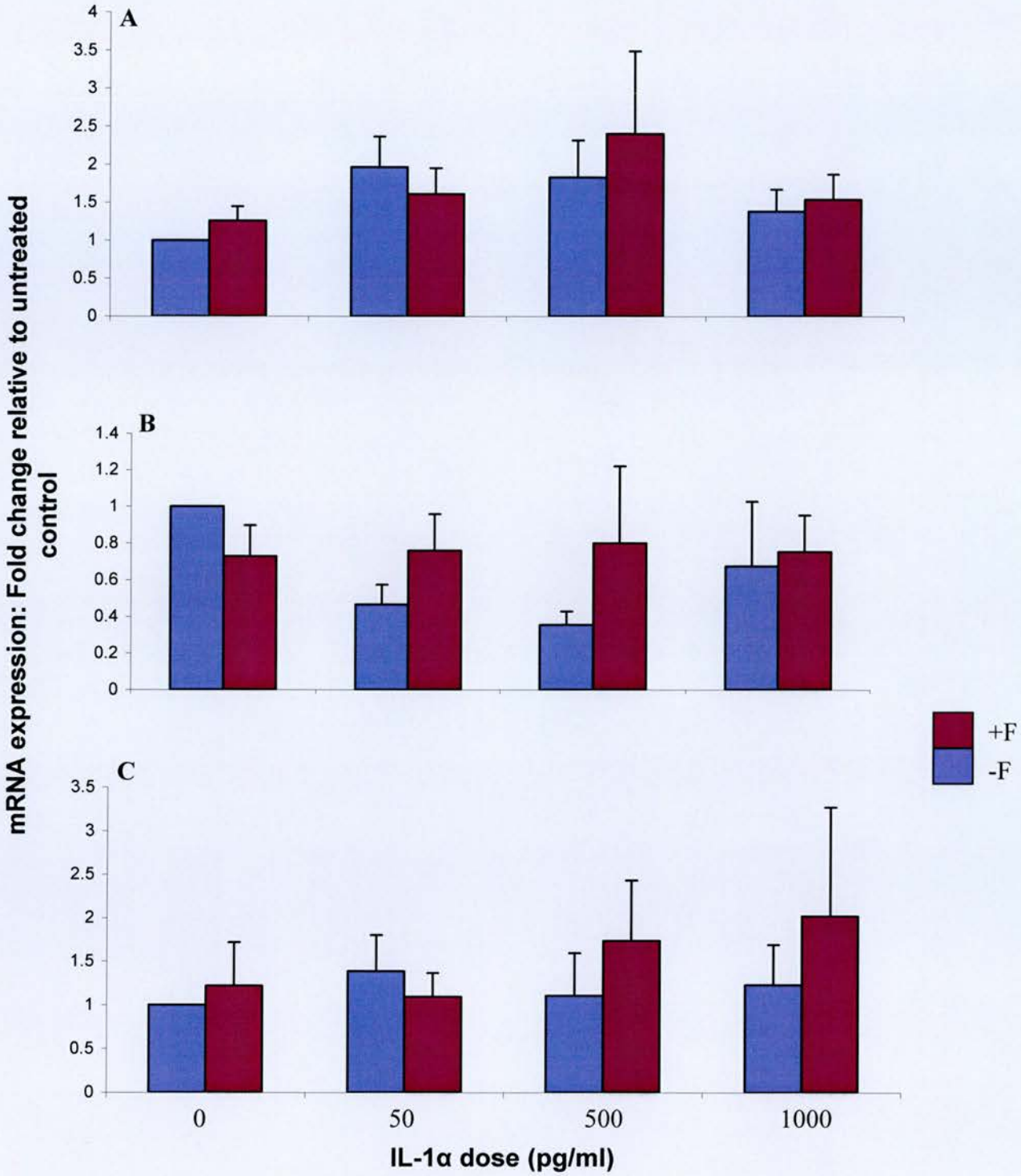


Figure 4.4 Expression of MR mRNA in response to increasing doses of IL-1α in the presence and absence of cortisol, mean ± SEM. RNA levels are measured as a fold change relative to untreated control. . A: endometrial stromal cells (n=5), B=epithelially enriched cells (n=4), C= mixed cells brushed from endometrial surface (n=5).

4.4 Discussion

There was a clear increase in 11βHSD-1 expression in endometrial stromal cells with IL-1α treatment. 11βHSD-1 expression was observed to increase steadily at doses up to 500pg/ml IL-1α, and did not increase significantly further with 1000pg/ml. Thus 500pg/ml IL-1α was found to be the optimal dose to induce 11βHSD-1 expression in agreement with studies on ovarian surface epithelial cells (OSE) by Yong et al. (2002), who found the same dose of IL-1α to be sufficient to induce 11βHSD-1 expression in human ovarian surface epithelial cells.

This increase in expression would lead to an increase in local cortisol availability in endometrial stromal cells. Cortisol could act on stromal cells in a paracrine manner to mediate inflammation in other cell types within the stroma, such as immune cells or component cells of the vasculature (endothelial and perivascular smooth muscle cells). Immune cells are often active at the same time as inflammatory molecules to fight infection or stress. Menstruation is a highly inflammatory process (Critchley et al., 2001b; Jabbour et al., 2006), and one in which the vasculature has a major role in the control of bleeding and restriction of blood loss. When progesterone is withdrawn and the endometrium receives the signal to prepare for menstruation, cytokine signalling induces increased vascular permeability (Kelly et al., 2002). During the first 24 hours of menstruation, arterioles undergo periods of vasoconstriction, as first demonstrated by Markee (1940). This in turn leads to hypoxia, which induces the release of a number of factors associated with inflammation such as prostaglandins. These factors are the signal for menstruation to begin (Sugino et al., 2004; Jabbour et

al., 2006). There is an association between activation of pro-inflammatory cytokines and release of MMPs in menstruation, however it is currently unclear whether this occurs before or after the vasoconstriction. It has been proposed that IL-1 has a role in the initiation of menstruation via the stimulation of MMPs and prostaglandins, (Cole et al., 1995; Kawano et al., 2001; Rossi et al., 2005). Thus it would be important to modulate the inflammatory process in these cells.

A similar pattern of expression of 11βHSD-1 mRNA was seen in epithelially enriched endometrial cultures following IL-1α and cortisol treatment. The increase in 11βHSD-1 was much greater in these epithelially enriched cells, a more than 50-fold increase over control following treatment with 500pg/ml IL-1α. The maximal responsive dose of IL-1α was again seen to be 500pg/ml. These data show that IL-1α appears to upregulate 11βHSD-1 mRNA expression in endometrial epithelial cells. Glands are secretory structures in the endometrium responsible for the proteins secreted by this tissue. Inflammation results in increased secretion of a number of growth factors, prostaglandins and other factors (Modugno et al., 2005). The glands are the site of endometrial secretion thus it follows that these secretory cell types may also be expected to express increased 11βHSD-1 leading to increased cortisol production. 11βHSD-1 protein has also been found to be predominantly expressed in the glands of the menstrual human endometrium as shown in 3.3.1 of this thesis. At key times of inflammation in the endometrium, for example implantation and menstruation, the glands undergo morphological changes and remodelling. In the process of decidualisation, the glands become atrophic and the cells lose their columnar shape. At menstruation the entire functional layer of the endometrium is

shed, the glands first elongating and then commencing to break up. Thus these cells undergo a large amount of stress in the course of the menstrual cycle. The anti-inflammatory action of cortisol is known to mediate the stress caused by an inflammatory response, thus it is important that a capacity to produce extra cortisol is available. The hypothesis that intracrine formation of cortisol in OSE cells limits inflammation and associated tissue injury at the time of ovulation (Yong et al., 2002; Hillier & Tetsuka, 1998) also applies to the human endometrium, lending weight to the proposal that epithelial cells behave similarly regardless of the tissue they are in, and in particular the tissues of the female reproductive tract undergo similar processes.

In a mixed population of cells containing both surface epithelial cells and stromal cells, with potentially some contaminating glandular epithelia also, there was once again an increase in the expression of 11βHSD-1 in response to increasing IL-1α dose. There was also an amplification of the response with the addition of cortisol. In these cells there was a small further increase in 11βHSD-1 expression with 1000pg IL-1α that was not seen in the other cell populations; however the increase seen with the addition of cortisol was not any greater at this dose of IL-1α than at 500pg/ml. Due to the monolayer nature of the surface epithelium it is extremely difficult with the methods available to get a population of solely surface epithelial cells, thus there is a great degree of variation between cultures that is reflected in the data. The surface epithelium is the site of inflammatory reactions. It is the first part of the functional endometrium to be sloughed off at menstruation and must be penetrated to allow implantation and embedding of a fertilised embryo. Thus it must also be the

site of increased local cortisol production resulting from increased 11βHSD-1 expression. There is also evidence of the feed-forward effect of cortisol discussed earlier at the surface of the endometrium. These data also mirror those of Rae et al. (2004), showing that similar processes are mediated in a similar manner at the surface of both the endometrium and the ovary.

All three cell types (epithelially enriched, stromal, and mixed cells from the endometrial surface) studied show a broadly similar trend in expression of 11βHSD-1 in response to IL-1α plus cortisol. As reported in other tissues, including the OSE (Escher et al., 1997; Feinstein & Schleimer, 1999; Yong et al., 2002; Rae et al., 2004a), IL-1α upregulates the expression of 11βHSD-1 and this effect is amplified by the addition of cortisol. As also reported by Rae et al. (2004a) in ovarian surface epithelium, the maximal dose of IL-1α to elicit a maximal response is 500pg/ml.

Despite the failure of cytokeratin staining procedures to identify epithelial cells, the huge scale difference in 11βHSD-1 expression in epithelia compared to fibroblast cultures is suggestive the IL-1α response occurs in the epithelia, and not fibroblasts. The effect seen in surface cells is at a much smaller scale than that in glandular epithelia, suggesting that the response is related to the location near the surface of the tissue rather than the cell composition of the samples.

No significant effect was seen on expression of 11βHSD-2 in response to IL-1α, cortisol, or IL-1α plus cortisol in either endometrial stromal cells or epithelia. Expression of 11βHSD-2 was relatively constant in these cells regardless of

treatment. There was also no effect of stage of the menstrual cycle as reported earlier in this thesis (3.3.2), and also in McDonald et al., (2006). Thus it appears that 11βHSD-2 in endometrium is maintained at stable levels and is not influenced by internal or external factors. Rae et al. (2004a) also found IL-1α in the presence or absence of cortisol to have no measurable effect on 11βHSD-2 expression in the OSE, following the hypothesis that epithelial cells of the female reproductive tract behave similarly regardless of tissue type.

11βHSD-2 protein has been studied across the menstrual cycle by Smith et al. (1997) and in 3.3.2 of this thesis (also McDonald et al., 2006). It was found to be predominantly expressed in the glandular and surface epithelial cells, with no significant variation across the menstrual cycle. It seems that 11βHSD-2 is not affected by fluctuating steroid hormone levels, or by regulatory factors such as cytokines which have been tested.

11βHSD-2 was not detectable by QRT-PCR in cells taken from the surface of the endometrium. This is the site that will experience the primary inflammatory response during tissue remodelling and thus there may be a requirement for the anti-inflammatory action of cortisol, but not for cortisone production. This corresponds to the findings of Yong et al. (2002) and Rae et al. (2004a) who discovered negligible expression of 11βHSD-2 in OSE cultures.

GR mRNA was expressed in all cell types studied, although there was no significant change in GR mRNA expression in the presence of IL-1α, cortisol or the

combination of the two, compared to untreated cells in endometrial stromal cultures. There was a trend towards increased GR mRNA in endometrial epithelial cells following treatment with 50pg/ml IL-1α. Addition of cortisol had no further effect, nor did increased doses of IL-1α. Although this result is not statistically significant, it is of interest when compared to the findings of Rae et al. (2004a) in studies in ovarian surface epithelium, who discovered a small but significant increase in GR mRNA expression in response to IL-1α treatment. This trend in expression was not replicated in cells from the endometrial surface, where no effect of either IL-1α, cortisol, or IL-1α plus cortisol was seen.

However studies in a number of other cells, such as T cells (Kam et al., 1993), liver cells (Falus and Beres, 1995; Stith and McCallum 1983; Hill et al., 1988) and lung cells (Liu et al., 1993; Verheggen et al., 1996) have shown treatment with cytokines including IL-1α to reduce GR expression and function. This seems to be a tissue-specific response, and Pariante et al. (2006) proposed that it provides a putative pathway for the immune system to influence the HPA axis and disease expression. In the female reproductive tract this reduction does not occur, in fact we see a trend towards increased GR expression following IL-1α treatment. This corresponds with the increased 11βHSD-1 expression seen in the same cells, increased GR expression may further sensitise the cells to the cortisol regenerated by 11βHSD-1. Perhaps GR in the female reproductive tract behaves differently in response to IL-1α to cells in other tissues due to the repetitive tissue injury and repair that occurs throughout the cycle, and the need for glucocorticoids to respond and modulate this response. However in endometrial cells there was only a small change in GR expression in

response to cytokine treatment, suggesting perhaps that regulation of the anti-inflammatory response of glucocorticoids by cytokines occurs at the pre-receptor level, in the form of modulation of 11βHSD-1 expression levels.

There is a correlation between repeated inflammatory events and diseases such as cancer in the female reproductive tract. It has been proposed by Gubbay et al. (2004; 2005) that the tissue injury and repair that occurs in the ovarian surface epithelium at each ovulation may contribute to the incidence of ovarian cancer. Gubbay et al. (2004) also found that ovarian cancer cell lines expressed higher levels of 11βHSD-2 than those taken from normal ovarian surface epithelium. Thus they proposed a correlation between increased 11βHSD-2 and subsequent reduction of local cortisol levels, and incidence of ovarian cancer (Gubbay et al., 2004). It may be postulated then that increased 11βHSD-1 at times of inflammation confers some protection to the tissue against cancer. A similar link between inflammatory processes and endometrial cancer was postulated by Modugno et al. (2005), and it follows from this that the increase in 11βHSD-1 and GR but not 11βHSD-2 seen in endometrial cells offers some protection to a tissue repeatedly exposed to inflammation.

MR mRNA expression did not differ significantly in the presence of IL-1α with or without the addition of cortisol in all 3 of the endometrial cell types studied (epithelially enriched, stroma, and cells from the surface of the endometrium). Although cortisol is a glucocorticoid, it binds with high affinity to the mineralocorticoid receptor also, making MR expression a relevant end-point for this study. As it was not affected by IL-1α or cortisol, it is suggested that the

mineralocorticoid pathway is regulated in a different manner to the glucocorticoid pathway in human endometrium.

There was a large degree of variation between samples in all of these studies. There is natural variation in levels of gene expression between patients; in this case this may be increased due to the fact the samples were collected opportunistically from patients at all stages of the menstrual cycle and some of the patients did not have regular cycles. Rae et al., (2004b), utilising microarray, found that after 3 weeks in culture in the presence of serum, any effect of circulating steroids at the time of collection was nullified, and this also appeared to be the case with the endometrial cells (based on results shown here and visual observations) however, the stage of the cycle when the sample was taken would still impact on the relative proportions of cell types. In epithelially enriched cell populations stromal cells had a tendency to become more dominant over time so care had to be taken to utilse cells before the epithelial cells were overgrown. Due to the opportunistic nature of sampling, both full-thickness endometrial biopsies and pipelle sampling were used, thus the size of samples varied. Care was taken to minimise this variation, but it was impossible to remove this effect completely.

Concluding remarks

These studies add weight to the hypothesis advanced by Yong et al. (2001) and Rae et al. (2004a) that cortisol operates a feed-forward regulatory mechanism, whereby it synergistically with IL-1α, upregulates its own production via 11βHSD-1 expression,

allowing cortisol regeneration at the local site of action in the current context in endometrial epithelial and stromal cells.

Overall, it has been shown that IL-1α promotes 11βHSD-1 expression in human endometrium, predominantly in the glandular epithelia, and has a small effect on GR expression in these cells. 11βHSD-2 and MR expression are not affected by this cytokine. These data are consistent with the report by Yong et al. (2002) and Rae et al. (2004a) in the ovarian surface epithelium, suggesting similarities between the two reproductive tissues, and lending weight to the theory that epithelial cells behave similarly regardless of tissue type.

Chapter 5:

Local regulation of sex steroid availability by steroid metabolising enzymes

5.1 Introduction

Sex steroid hormones are vitally important in the regulation of events in the female reproductive tract. Both estradiol and progesterone are key, along with a number of androgens including testosterone and DHEA. Sex steroids are secreted by the ovary and adrenal gland and transported to target sites in the blood. In addition to these steroids however, there is tight regulation of sex steroid availability and action at a local, pre-receptor level. A number of metabolic enzymes are involved in this local regulation of sex steroids, including the related 3βHSD, 3αHSD (AKR1C) and 17βHSD dehydrogenase subfamilies.

3βHSDs are involved in the conversion of pregnenolone to progesterone, a key step in the synthesis of estrogens, androgens, mineralocorticoids and glucocorticoids (Readhead et al., 1993; Rhee et al., 2003). They are also involved in the metabolism of a number of androgens. The reactions catalysed by 3βHSD are given in Table 5.1, and illustrated in the pathway diagram in section 1.2. 3βHSD expression in the endometrium has been studied by Tang et al. (1993) who found the enzyme to be active in endometrium, and by Rhee et al. (2003) who found 3βHSD to be expressed throughout the menstrual cycle, with expression levels increasing in the secretory phase.

The AKR1C and 17βHSD enzymes are involved in the regulation and metabolism of sex steroids. The reactions performed by the four AKR1C enzymes are shown in Table 5.1.

Table 5.1 Sex steroid metabolising reactions of enzymes discussed in this chapter.

Enzyme	Substrate	Product
3βHSD	Pregnenolone DHEA Androstenediol DHT	Progesterone Androstenedione Testosterone Androstanediol
AKR1C1	Progesterone	20α-hydroxyprogesterone
AKR1C2	DHT Androstane-3β, 17β-diol	Androstanediol DHT
AKR1C3	Androstenedione Estrone DHT Progesterone Androstanediol Androstanediol Deoxycortisol	Testosterone Estradiol Androstanediol 20α-hydroxyprogesterone DHT Androsterone 20α-dihydrodeoxycortisol
AKR1C4 (in liver)	5α/5β-dihydrosteroids	5α/5β-tetrahydrosteroids
17βHSD-2	Estradiol Testosterone	Estrone Androstenedione

The expression of AKR1C enzymes in endometrium has been investigated, but not in detail. All four AKR1C enzymes were found to be expressed in the uterus at low levels (Penning et al., 2000), and AKR1C3 (17βHSD-5) has been shown to be expressed in both the proliferative and secretory phases (Ito et al., 2006). 17βHSD-2 protein is expressed in the glandular epithelia in the secretory endometrium, and at negligible levels in the proliferative phase (Casey et al.; 1994, Burton et al., 2003). 17βHSD-2 mRNA expression however shows the opposite pattern, with expression greatest in the proliferative phase (Casey et al.; 1994, Mustonen et al.; 1998, Burton et al., 2003). A

possible explanation for this was given by Burton et al. (2003) of enhanced stability of mRNA, resulting mRNA persisting for extended periods without being translated.

Expression of the sex steroid receptors in the endometrium has been well characterised and reviewed earlier in section 1.2.4 of this thesis. PR and AR are expressed at low levels in the proliferative phase of the cycle and upregulated in the secretory phase (Slayden et al., 2001; Lessey et al., 1988). ERα and ERβ on the other hand, are both found at higher levels in proliferative endometrium (Lessey et al., 1988; Critchley et al., 2001a). AR is localised to the stromal cells, whereas ERα and PR are predominantly glandular. ERβ is also found in the endothelial cells, where ERα is not (Critchley et al., 2001a).

The aims of this chapter were to study the expression patterns of 3βHSD and AKR1C1-4 in endometrium across the menstrual cycle and in first trimester decidua, using both mRNA and protein detection techniques.

5.2 Materials and Methods

5.2.1 Tissue Collection and Subjects

Local ethical approval and informed patient consent were provided for all samples collected. Samples were collected as described in section 2.1. Briefly, 18 endometrium-only biopsies were collected at hysterectomy or by pipelle sampling device and used for the extraction of RNA. Five first trimester decidua biopsies were collected at elective surgical termination of pregnancy and used for RNA extraction.

34 full-thickness endometrial biopsies were collected at hysterectomy and wax embedded for use in immunohistochemistry. Five first trimester decidua biopsies were collected at elective surgical termination of pregnancy and also wax embedded for immunohistochemistry.

All subjects providing samples were of reproductive age, with regular menstrual cycles of 25-35 days and had no exogenous hormone treatment in the previous 3 months. Tissue samples were consistent across three parameters: date of reported last menstrual period, histological stage and ovarian stage of cycle as determined by serum estradiol and progesterone concentrations at the time of biopsy, measured by RIA.

The human tissue samples used in this chapter have been previously detailed in Tables 3.1 and 3.2.

5.2.2 RNA extraction and reverse-transcriptase PCR

RNA was extracted using methods described in 2.3.2. 1μl was used to measure RNA concentration and quality using an Agilent 2000 Nano Bioanalyser as described in section 2.3, and the remaining RNA was stored at -70°C.

Reverse transcriptase-PCR was performed using standard conditions as described in section 2.4.

5.2.3 Quantitative Real-Time PCR (QRT-PCR)

Oligonucleotide forward and reverse primers and oligonucleotide Taqman probes were used to detect the sequences of interest. The probes used were commercially available pre-validated from Applied Biosystems Assay on Demand. The reference numbers of the primer/probe sets are shown in Table 5.2.2. Quantitative Real-time PCR was performed using a 7900 sequence detection system and analysed as described in section 2.5.

Table 5.2 Taqman primers and probes

Gene	Reference Number
3βHSD-1	Hs00426435_m1
3βHSD-2	HS00605123_m1
AKR1C1	Hs00912746_m1
AKR1C2	Hs00912742_m1
AKR1C3	Hs00366267_m1
AKR1C4	Hs00559542_m1

Sequences are not available but primer/probe sets were pre-validated by ABI.

Analysis of data and statistical analysis was performed as described in section 2.7.

5.2.4 Immunohistochemistry

Immunohistochemistry was performed using antibodies specific to 3βHSD and AKR1C3 by standard methods as described in Chapter 2 using biotin-conjugated secondary antibodies and ABC-Elite avidin biotin peroxidase complex (Vector). Immunoreactivity was detected using the chromagen 3,3'-diaminobenzidine (DAB).

The 3βHSD antibody was a rabbit polyclonal raised against human recombinant 3βHSD-2 previously in the laboratory. Due to the high similarity between the two 3βHSD isoforms, it detects both 3βHSD-1 and 3βHSD-2. The AKR1C3 mouse

monoclonal antibody was kindly provided by Prof. Trevor Penning, University of Pennsylvania (Lin et al., 2004). Semi-quantitative visual analysis and statistics were performed as described in Chapter 2. The individual conditions for each antibody as determined by titration are shown in Table 5.3.

Table 5.3 Immunohistochemical conditions

Primary Antibody	Dilution	Secondary Antibody	Antigen Retrieval	Control
3βHSD	1:500	Goat anti-rabbit, 1:200	Not required	+ve: human placenta; -ve: pre-immune serum
AKR1C3	1:200	Horse anti-mouse, 1:200	Pressure cooking in 0.01M NaCitrate, pH6, 5 minutes.	+ve: human kidney; -ve: matched IgG

5.3 Results

5.3.1 3βHSD-1 mRNA expression in human endometrium

3βHSD-1 mRNA was not detectable by QRT-PCR in human endometrium at any stage of the menstrual cycle. The transcript was however expressed in first trimester decidua. These data are shown in Figure 5.1.

5.3.2 3βHSD-2 mRNA expression in human endometrium

3βHSD-2 mRNA was not detectable after 40 cycles of QRT-PCR in non-pregnant human endometrium or first trimester decidua.

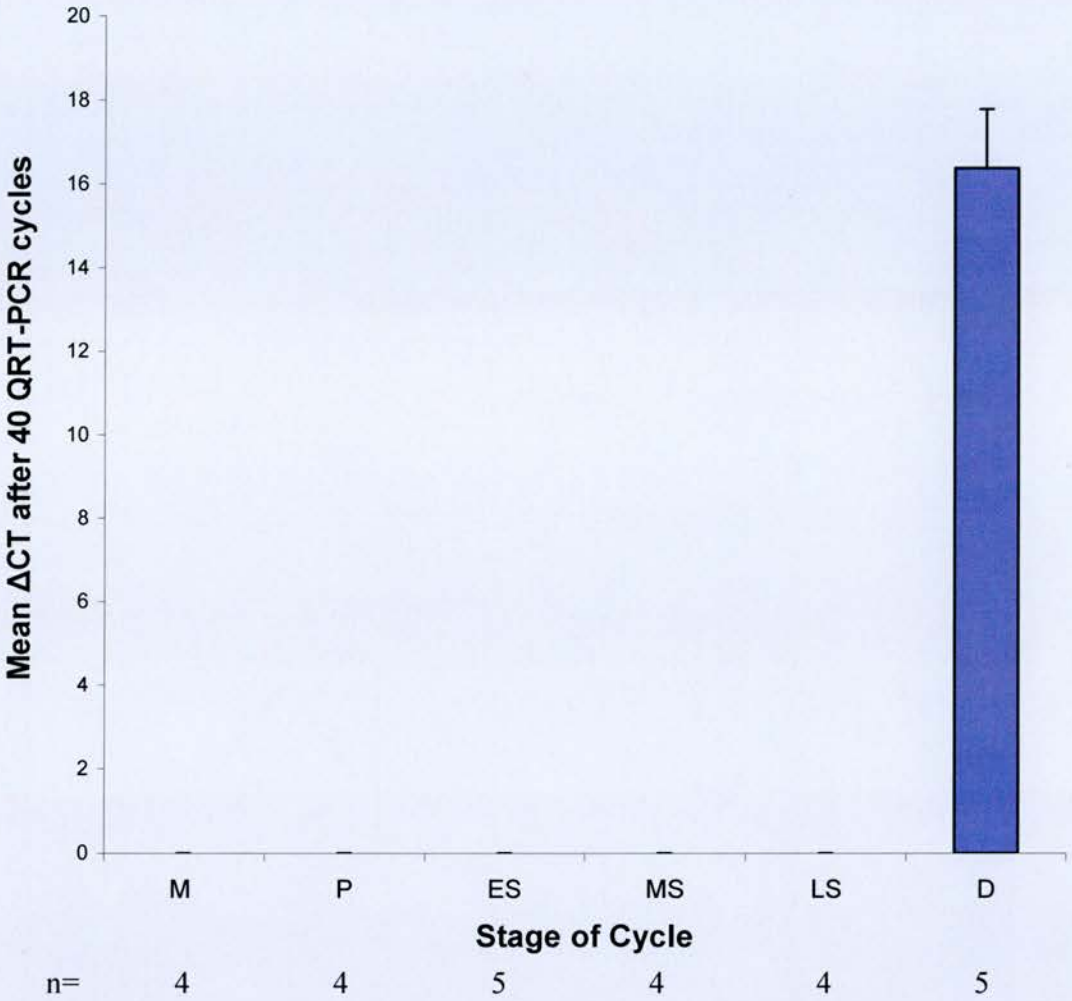


Figure 5.1 Expression of 3βHSD-1 mRNA across the menstrual cycle and in first-trimester decidua, mean ± SEM M = menstrual, P = proliferative, ES = early secretory, MS = mid secretory, LS = late secretory, D = decidua. As no mRNA was detectable in any samples other than first trimester decidua it was not possible to compare samples, so data are represented as mean "ΔCT" values (CT for 3βHSD1-CT for 18S) as described in 2.5.2. Statistical analysis was not possible as 3βHSD-1 was not detectable in any samples other than decidua.

5.3.3 3βHSD protein expression in human endometrium

3βHSD protein was detected using an antibody raised against recombinant human 3βHSD2 as described in 5.2.4; however this antibody similarly detects both 3βHSD-1 and -2.

3βHSD protein was localised predominantly to the glandular and surface epithelia, with some expression in the endothelial cells of the vasculature. Expression of 3βHSD protein was very low at all stages of the menstrual cycle in non-pregnant endometrium and did not differ significantly between the functional and basal layers of the endometrium.

In first trimester decidua, immunostaining was stronger than that observed in non-pregnant endometrium. Localisation was again primarily located to the glandular and surface epithelia and vasculature; however there were also low levels of 3βHSD protein expressed in the stroma.

These data are illustrated in Figures 5.2 and 5.3. Charts showing immunoscores are included in Figure A5 of Appendix 1.

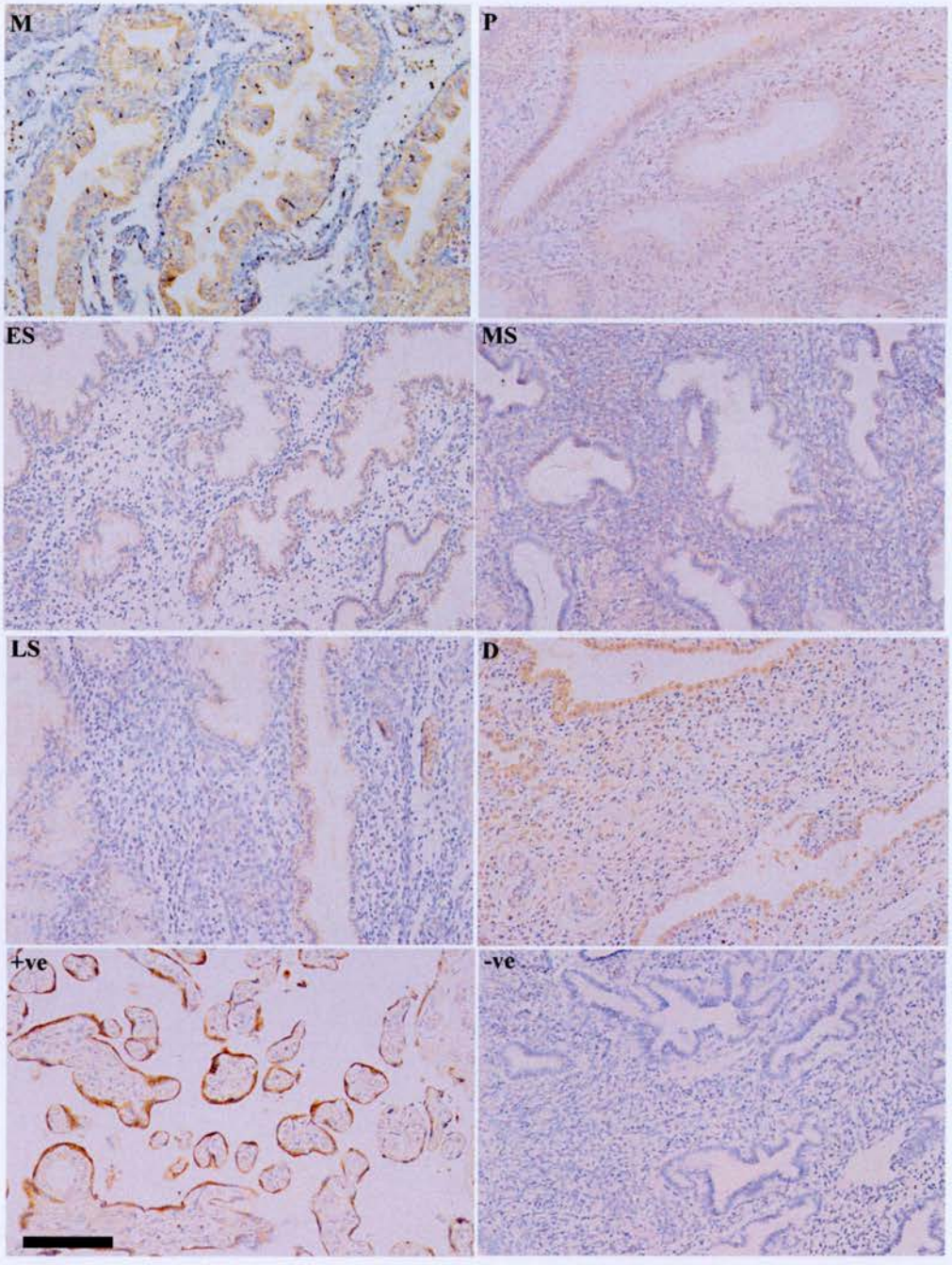


Figure 5.2 Immunolocalisation of 3βHSD protein in the functional layer of the endometrium and decidua. M = menstrual, P = proliferative, ES = early secretory, MS = mid secretory, LS = late secretory, D=decidua, +ve= positive control, human placenta, -ve=negative control, pre-immune serum. Scale bar=10 microns.

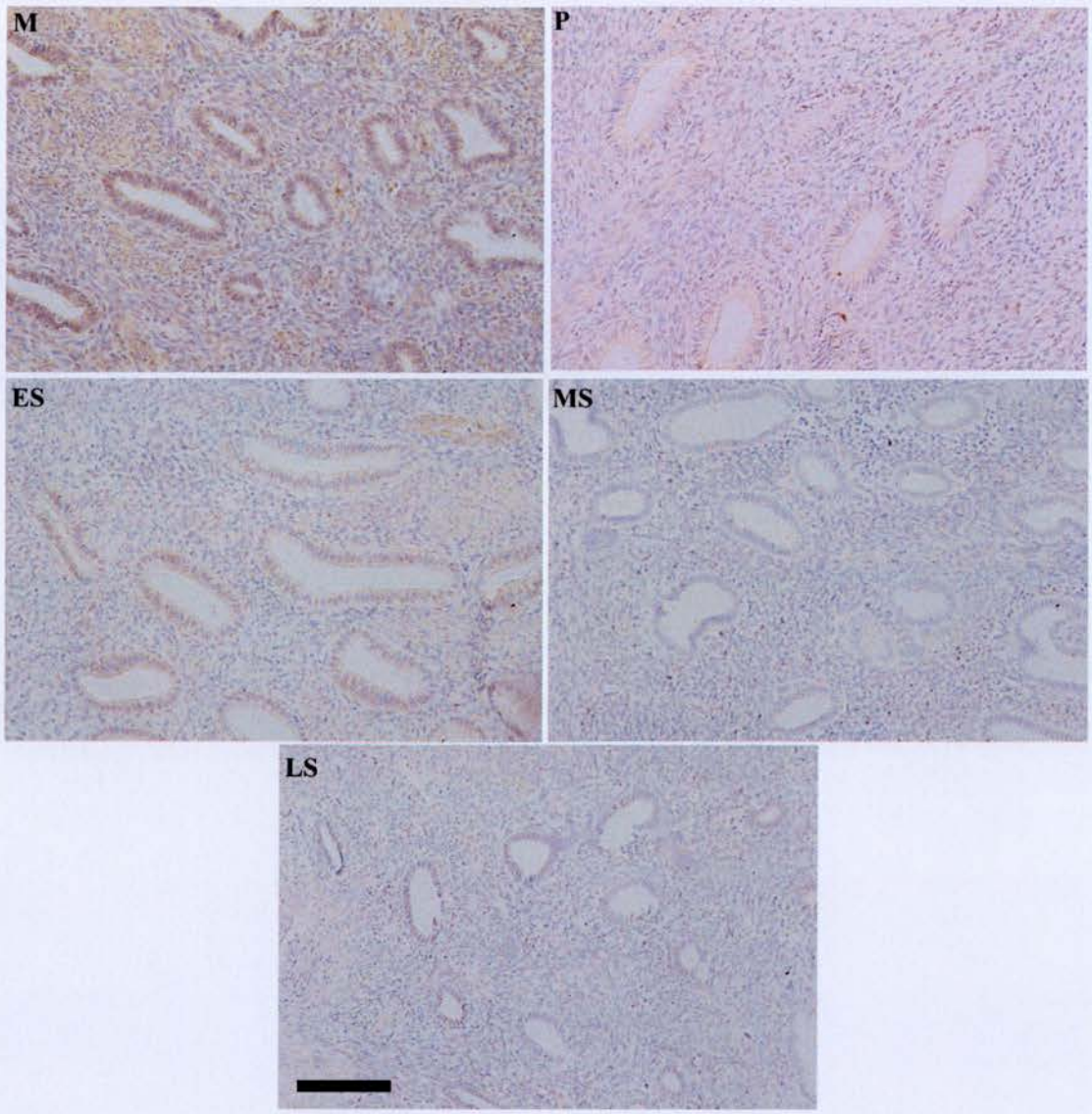


Figure 5.3 Immunohistochemical localisation of 3βHSD in the basal layer of the endometrium. M = menstrual, P = proliferative, ES = early secretory, MS = mid secretory, LS = late secretory. Scale bar=10 microns.

5.3.4 AKR1C mRNA expression in human endometrium

AKR1C1 mRNA was expressed in human endometrium at all stages of the menstrual cycle, with the apparent greatest expression in the secretory phase, but this difference was not found to be statistically significant. AKR1C1 mRNA was also expressed in first trimester decidua at a low level, although not significantly lower than that in non-pregnant endometrium.

AKR1C2 mRNA was also expressed in normal endometrium at all stages of the menstrual cycle. Greatest expression was seen in the mid secretory phase, with very low levels of expression in the late secretory stage. Again, these differences in expression were not found to be statistically significant. AKR1C2 mRNA was expressed at low levels in first trimester decidua.

AKR1C3 mRNA was expressed at all stages of the menstrual cycle. Expression was significantly greater in the early secretory stage of the cycle than at any other stage. Expression levels decreased from early to late secretory phases. AKR1C3 mRNA was expressed at very low levels in first trimester decidua.

AKR1C4 mRNA was not detectable at any stage of the menstrual cycle or in first trimester decidua after 40 cycles of QRT-PCR.

These data are shown in Figure 5.4

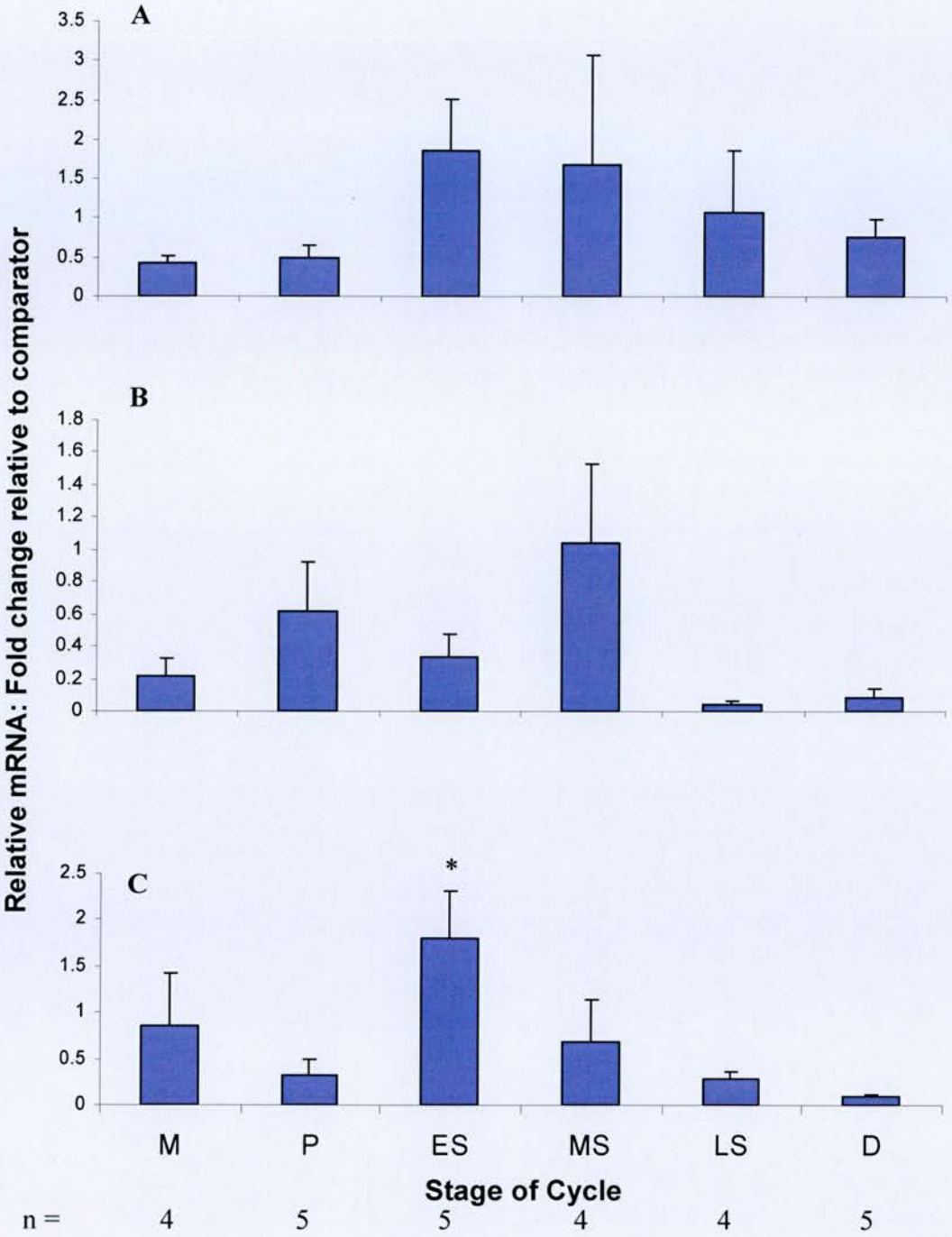


Figure 5.4 Expression of AKR1C1-1C3 mRNA across the menstrual cycle and in first-trimester decidua, mean ± SEM. M = menstrual, P = proliferative, ES = early secretory, MS = mid secretory, LS = late secretory, D = decidua). A=AKR1C1, B=AKR1C2, C=AKR1C3. The comparator was a proliferative endometrium sample. *p<0.05 compared to other stages of the menstrual cycle.

5.3.5 AKR1C3 protein expression in human endometrium

AKR1C3 protein was strongly expressed across the menstrual cycle. The protein was localised to the glands, surface epithelia and was strongly expressed in the endothelial cells of the vasculature. Expression was similar in both the functional and basal layers of the endometrium and did not differ significantly across the menstrual cycle. A similar intensity and pattern of immunostaining was observed in first trimester decidua.

These data are shown in Figure 5.5 and 5.6. Charts showing immunoscores are included in Figure A6 of Appendix 1.

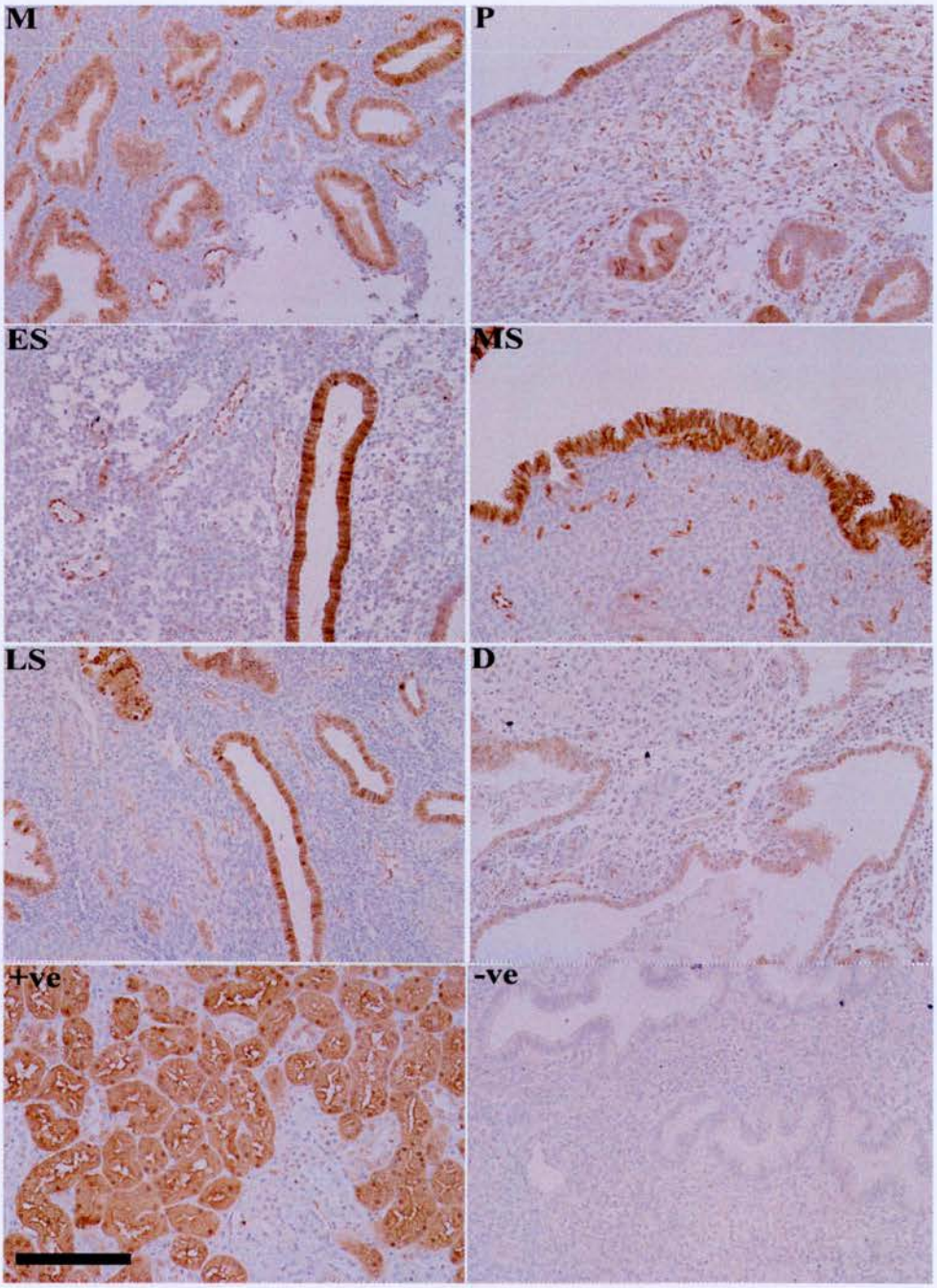


Figure 5.5 Immunolocalisation of AKR1C3 (17βHSD-5) protein in the functional layer of the endometrium and decidua. Endothelial cells of the vasculature are indicated with an arrow). M=menstrual, P=proliferative, ES=early secretory, MS=mid-secretory, LS=late secretory, D=first trimester decidua, +ve= positive control, human kidney, -ve=negative control, primary antibody substituted for matched IgG. Scale bar=10 microns.

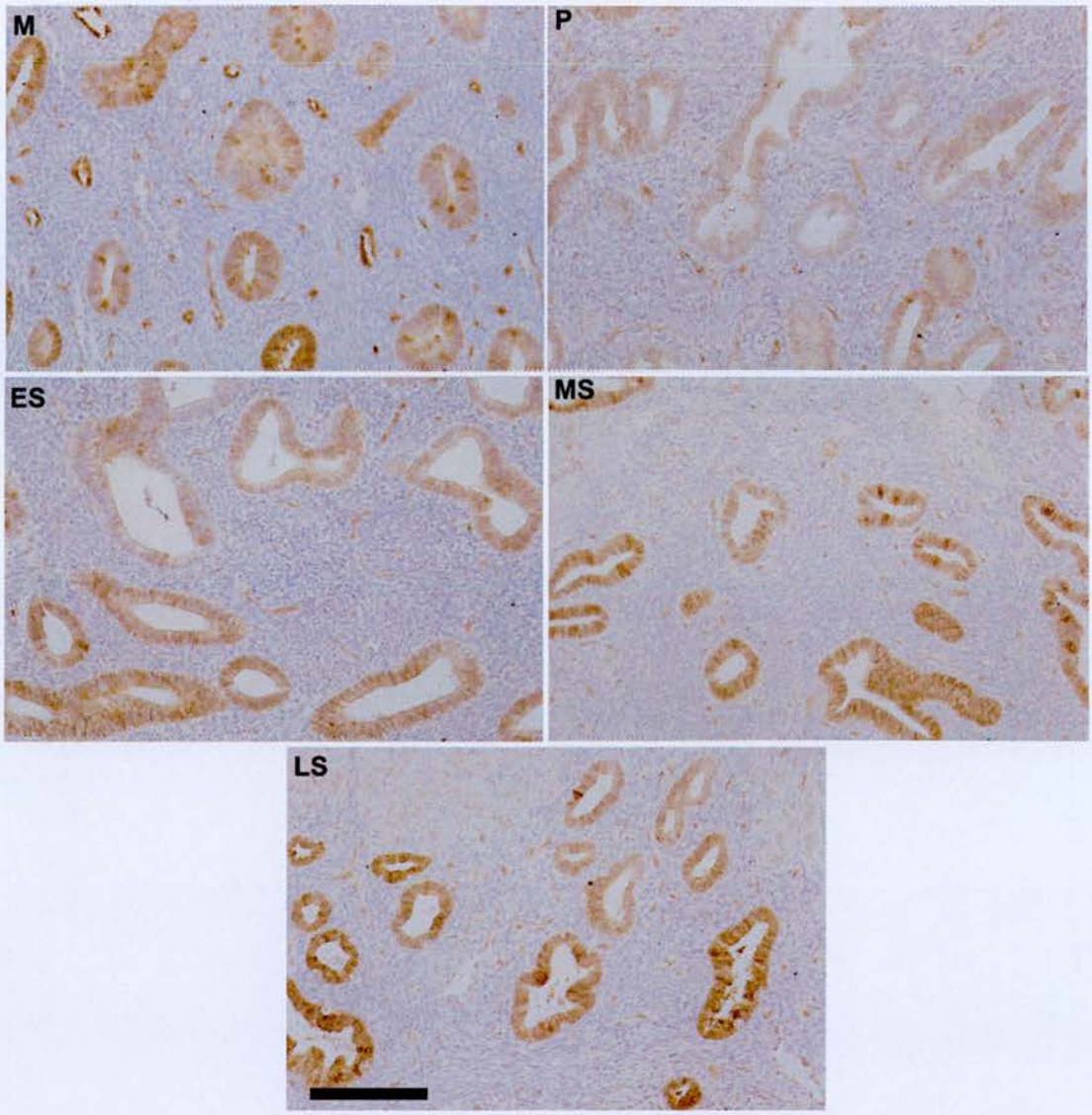


Figure 5.6 Immunohistochemical localisation of AKR1C3 (17βHSD-5) in the basal layer of the endometrium. M = menstrual, P = proliferative, ES = early secretory, MS = mid secretory, LS = late secretory. Scale bar=10 microns.

5.3.6 Summary of Protein Expression Data

Table 5.4 Functional layer of normal endometrium

	Glands	Stroma	Surface Epithelia	Endothelium	Perivascular Cells
3βHSD	+/-	-	+/-	-	-
AKR1C3 (17βHSD-5)	++	-	++	++	-

Table 5.5 Basal layer of normal endometrium

	Glands	Stroma	Surface Epithelia	Endothelium	Perivascular Cells
3βHSD	+/-	-	+/-	-	-
AKR1C3 (17βHSD-5)	++	-	++	++	-

Table 5.6 First trimester decidua

	Glands	Stroma	Surface Epithelia	Endothelium	Perivascular Cells
3βHSD	+	-	+	+/-	+/-
AKR1C3 (17βHSD-5)	++	-	++	++	-

++ intense immunostaining
+ moderate immunostaining
+/- mild immunostaining
- no immunostaining

5.4 Discussion

Neither 3βHSD-1 nor -2 mRNA was detectable in human endometrium. However 3βHSD-1 mRNA was found to be present in first trimester decidua. Immunohistochemical studies showed 3βHSD protein to be predominantly localised to the epithelial cells, expressed at very low levels in normal human endometrium and increased in first trimester decidua. These data are consistent with data published by Rhee et al. (2003) who found 3βHSD to be expressed at low levels in proliferative endometrium, with a small increase in the secretory phase and a greater upregulation in first trimester decidua. In the present study, expression patterns have been investigated across the menstrual cycle differentiating between early, mid and late secretory stages. It has also been shown here for the first time that 3βHSD protein is expressed at low levels in menstrual endometrium.

Rhee et al. (2003) also performed RT-PCR to suggest that the 3βHSD isoform present was 3βHSD-1. The results of the quantitative real-time PCR shown here suggest this is the case, as 3βHSD-1 mRNA is the only transcript to be expressed in first trimester decidua, where the most substantial protein expression is observed.

3βHSD expression was seen to be greatest in the early stages of pregnancy. If pregnancy is detected, progesterone levels are maintained at the increased levels seen in the secretory phase. As 3βHSD is responsible for progesterone synthesis, this suggests there

is perhaps a feed-forward mechanism in action whereby progesterone upregulates its own formation.

Rhee et al. (2003) also proposed that endometrial progesterone may be derived from two sources. As well as circulating progesterone secreted in the "classical" manner by the ovary, their report of the presence of P450scc enzyme involved in pregnenolone formation in human endometrium suggests an alternative local source of progesterone from local 3βHSD action. This additional source of progesterone becoming available in early pregnancy may account for the maintenance of elevated progesterone levels required for pregnancy. Additionally, local progesterone may be necessary to ensure progesterone-dependent genes are appropriately expressed.

3βHSD has additional activities besides progesterone formation, including the metabolism of androgens, in particular of 5αDHT, 5α-androstanediol and DHEA. The activation or inactivation of androgens, depending on available cofactors, affects the local steroid balance. The presence of 3βHSD, as well as other steroidogenic enzymes could facilitate the endometrium to produce its own steroids, with 3βHSD acting both early (conversion of pregnenolone to progesterone is a key step in formation of almost all steroid hormones) and later in the pathway of androgen synthesis.

AKR1C1 mRNA was expressed throughout the menstrual cycle, with greatest expression in the early and mid secretory phases. The predominant activity of this enzyme in intact cells is the reduction of progesterone to 20α-hydroxyprogesterone

(20αOHP), thus deactivating progesterone. Low levels of AKR1C1 would thus be expected in first trimester decidua, to maintain the elevated progesterone levels required for successful establishment of pregnancy. Levels of AKR1C1 mRNA were indeed lower in first trimester decidua than in the secretory endometrium. Lowest mRNA levels were seen in menstrual and proliferative endometrium. This is also as would be expected, since estrogen induces glandular proliferation and progesterone has an inhibitory action (Rizner et al., 2006). This is the stage of the menstrual cycle in which the functional layer of the endometrium is regenerated following shedding at menstruation.

AKR1C1 levels have been shown to be elevated in endometrial cancer (Rizner et al., 2006), thereby keeping local progesterone levels low and removing the inhibitory effect of progesterone on cell proliferation.

Highest levels of AKR1C1 were seen in the early and mid secretory stages of the cycle, the time when progesterone expression is greatest. AKR1C1 could be acting here to modulate progesterone formation and ensure low levels of this steroid are maintained.

AKR1C2 mRNA was expressed across the menstrual cycle; levels were low at all stages of the cycle, with reduced expression in late secretory endometrium and first trimester decidua and greatest expression in the proliferative and mid secretory phases. The predominant activity of this enzyme in intact cells is the inactivation of DHT to androstanediol, a potent androgen converted a less potent form of the steroid. Thus, this

enzyme has been described as an "off switch" for the androgen receptor (Penning et al., 2004). The androgen receptor is expressed in greatest levels in the proliferative phase and a significant decline is seen in the late secretory phase, corresponding with the decrease in the enzyme that makes available active ligand.

AKR1C3 (17βHSD-5) mRNA was expressed at all stages of the menstrual cycle. Expression was significantly upregulated in the early secretory stage of the cycle in comparison with other stages, thereafter levels decline in the mid and late secretory stages of the menstrual cycle respectively, before dropping to its lowest levels in first trimester decidua.

The availability of a specific AKR1C3 (17βHSD-5) antibody allowed protein localisation to be studied in the endometrium. Intensity of immunostaining did not vary across the menstrual cycle. AKR1C3 (17βHSD-5) protein was strongly expressed in the glandular and surface epithelia, and endothelial cells of the vasculature. Estrogen receptor β (ERβ), but not estrogen receptor α (ERα) has been shown to be expressed in these cells (Critchley et al., 2001a). This co-localisation suggests the potential prominent role for this enzyme in the endometrium is in estrogen metabolism, forming estradiol from inactive estrone.

The localisation of this enzyme to the vasculature also implicates a role in the regulation of endometrial bleeding. AKR1C3 (17βHSD-5) can also act to reduce progesterone to 20αOHP and thus may be implicated as a contributory factor in the progesterone

withdrawal associated with the late secretory stage of the cycle leading to menstruation, alongside the predominant source of progesterone withdrawal, luteal regression.

Both androgen and estrogen metabolism could be implicated in the low levels of AKR1C3 (17βHSD-5) mRNA seen in first trimester decidua – high progesterone levels must be maintained so conversion to 20αOHP is minimised. Additionally, progesterone must not be opposed by estrogen, thus estradiol synthesis via AKR1C3 (17βHSD-5) will also be restricted.

This is the first time AKR1C3 (17βHSD-5) has been studied in any depth in the human endometrium. A previous report b Pelletier et al. (1999) localised the enzyme to the glandular and surface epithelium but did not consider the expression of this enzyme across the different stages of the menstrual cycle, nor did they report any details of expression in endometrial vasculature.

AKR1C4 mRNA was not detectable in either human endometrium or first trimester decidua. This confirms previous reports (Penning et al., 2003) that this enzyme is largely liver-specific and acts to protect the liver from excess circulating hormones.

Previous studies (Burton et al., 2003) showed 17βHSD-2 mRNA to be expressed at greatest levels in the proliferative phase and downregulated in early secretory endometrium in a pattern reciprocal to that shown here for AKR1C3 (17βHSD-5). This suggested the possibility of a "switch" in operation in human endometrium, controlling

the expression of active androgens and estrogens via 17βHSD action. This trend was however not observed at a protein level. Burton et al. (2003) reported 17βHSD-2 protein expression only in the secretory endometrium; and it has been shown here that AKR1C3 (17βHSD-5) protein is expressed at all stages of the cycle. Additionally, a recent gene array study showed 17βHSD-2 to be highest in the mid secretory phase (Talbi et al., 2006).

Previous observations of expression have reported opposing results, with low levels of 17βHSD-2 in proliferative endometrium (Casey et al., 1994). From this we cannot clearly state that there is a distinct "on-off" switch between active and inactive estrogens and androgens operated by 17βHSD-2 and AKR1C3 (17βHSD-5), but a variable one. Control of these steroid hormones is likely to be more complicated and involve a number of other regulatory factors.

The metabolising enzymes discussed thus far regulate the availability of ligands for the sex steroid receptors. 3βHSD acts to create progesterone, the ligand for the progesterone receptor. 3βHSD is expressed in first trimester decidua, mainly in the glandular epithelia. PR is also expressed in first trimester decidua, but is localised to the stroma. In order for progesterone locally produced by 3βHSD to act via PR, it must first be transported to the stromal cells and thus acts in a paracrine signalling manner.

3βHSD is also able to act to metabolise androgens and AR is also expressed in the stromal compartment of first trimester decidua (Milne et al., 2005). Thus, the cognate receptors for the products of both types of 3βHSD action are present.

AKR1C1 acts to inactivate progesterone by converting it to 20α-hydroxyprogesterone. It is expressed at greatest levels in the secretory endometrium. Levels of circulating progesterone are greatest at this stage; PR is also expressed in endometrial stromal cells in the secretory phase of the cycle. This could further support the suggestion that AKR1C1 is acting to protect the endometrium from excessive progesterone action.

On the other hand, AKR1C2 is involved in androgen metabolism; producing the potent androgen DHT. Its expression is also greatest in the mid secretory endometrium, with a decline in the late secretory phase.

AKR1C3 (17βHSD-5) is perhaps the most "promiscuous" of the AKR1C enzymes, with the ability to activate estrogen, activate and inactivate androgens, and inactivate progesterone. Its expression peaks in early secretory endometrium. At this stage in the cycle, all three sex steroid receptors are expressed and levels of all three decline as the cycle progresses, a trend mirrored by AKR1C3 (17βHSD-5). This indicates that AKR1C3 (17βHSD-5) does indeed have the potential to modulate the expression of all three types of sex steroid in the human endometrium and indeed may perform more than one function in this tissue. Furthermore, AKR1C3 (17βHSD-5) very effectively converts deoxycortisol (DOC) to 20αDOC, ie inactivating the potent mineralocorticoid. This may also impact in the regulation of MR in the endometrium, as discussed in chapter 3 of this thesis.

Concluding Remarks

In this chapter it has been shown that 3βHSD expression is very low (undetectable by QRT-PCR and very mild immunoreactivity) in non-pregnant endometrium and expressed in first trimester decidua. AKR1C1-3 were all found to be expressed across the menstrual cycle, with AKR1C3 (17βHSD-5) mRNA significantly upregulated in the early secretory phase. This enzyme showed strong immunoreactivity in glandular and surface epithelia and endothelial cells. The expression profiles of these enzymes suggest an ability of the endometrium to produce and regulate the balance of local steroids, which in conjunction with the cyclical modulation of sex steroid receptors allow careful local control of sex steroid action

Chapter 6:

Effects of exogenous steroid administration (levonorgestrel) on pre-receptor signalling in human endometrium

6.1 Introduction

Both natural and synthetic steroids have a number of applications in many clinical situations, including contraception, hormone replacements and management of menstrual complaints. Levonorgestrel (LNG) is a widely used synthetic progestogen in contraceptives and is often administered through use of the levonorgestrel intrauterine system (LNG-IUS).

Levonorgestrel is a potent synthetic progestogen which also has significant androgenic activity. It is a widely used and highly effective contraceptive when used in the LNG-IUS. This intra-uterine system is also often used for management of menstrual disorders such as heavy menstrual bleeding (menorrhagia) (Critchley, 2003; Guttinger & Critchley, 2007).

A common side effect of many progestogen-only contraceptives is that of breakthrough bleeding; that is unscheduled bleeding between menstrual periods (Milling-Smith & Critchley, 2005). Breakthrough bleeding is a major reason for discontinued usage of the LNG-IUS by many women (Findlay, 1996; cited Milling Smith & Critchley, 2005).

However, the exact mechanism of breakthrough bleeding is as yet unknown. A great deal of the available evidence points to increased fragility of endometrial blood vessels, changes in local steroid responses and local angiogenesis mediators being some of the

factors involved (Milling-Smith & Critchley, 2005). Suggestions are that breakthrough bleeding occurs from a different vascular source to normal menstrual bleeding; from superficial dilated vessels (Hickey et al., 1996). Vascular fragility may also be increased (Song et al., 1995). The mechanism leading to aberrant angiogenesis and vascular fragility is not yet known.

Continuous exposure to an exogenous source of progestogen brings about a number of morphological changes in the endometrium. The endometrium takes on the appearance of first trimester decidua and is often described as pseudo-decidualised. In particular, the stromal cells become enlarged and oedematous and the glandular epithelia lose their cuboidal shape, flatten, and become atrophic (Phillips et al., 2003). These changes can be seen within one month of insertion of the LNG-IUS, but further changes are rare with continued usage. Within 1-3 months of removal of the LNG-IUS, endometrial morphology returns to normal and users return to complete fertility (Andersson et al., 1992; Silverberg et al., 1986). Pseudo-decidualised endometrium also expresses a number of markers of decidualisation such as insulin-like growth factor binding protein-1 (IGFBP-1) and prolactin (Critchley, 2003).

There are also reports that long-term use of the LNG-IUS can lead to altered patterns of sex steroid receptor expression, which are discussed further in section 1.3.1 of this thesis. Levonorgestrel can be metabolised by the same enzymes responsible for progesterone and androgen metabolism (Lemus et al., 1992). The effects of this

metabolism may control the binding affinity for LNG to AR and PR (Lemus et al., 1992).

The aim of this study was to investigate the expression patterns of a number of steroid metabolising enzymes and receptors in the endometrium of women using the LNG-IUS. The following end-points were studied at both mRNA and protein levels.

- 11βHSD-1 and -2
- GR and MR
- 3βHSD-1 and -2
- AKR1C1, 1C2 (mRNA only) and 1C3 (17HSD-5)

6.2 Methods

6.2.1 Subjects and endometrial tissue resource.

Local ethical approval and informed consent was obtained from all subjects.

Biopsies were collected by pipelle sampling device from seven women as described in section 2.1. These women had regular menstrual cycles of 25-35 days and had a LNG-IUS in situ, with no other hormonal intervention in the preceding 6 months. These samples were used for RNA extraction and QRT-PCR.

Biopsies were collected in a similar manner from a group of women attending clinic to have a LNG-IUS fitted. These women also had regular cycles of 25-35 days and no hormonal intervention for the preceding 6 months. Biopsies were taken in the proliferative (n=5) and secretory (n=5) stages of their cycles prior to insertion of the LNG-IUS, then follow up endometrial biopsies were collected at 1, 3, 6 and 12 months following insertion (n=5 at each point). These biopsies were fixed in NBF and used for immunohistochemistry.

These samples were collected for a previous study (Burton et al., 2003) and as there was insufficient material in this archival resource to complete all the studies required here, a further group of samples were used. These samples were collected from 5 women as

described above (samples for RNA extraction). A single biopsy was collected from each subject, who had a LNG-IUS in situ for varying periods of time. These samples were fixed in NBF and used in immunohistochemistry.

6.2.2 RNA extraction and reverse-transcriptase PCR

RNA extraction and reverse-transcriptase PCR were performed as described in sections 2.3 and 2.4.

6.2.3 Taqman Quantitative Real-Time PCR

QRT-PCR was performed using standard methods as described in section 2.5. Oligonucleotide forward and reverse primers, and oligonucleotide Taqman probes were used to detect sequences of interest. The probes were commercially available from Applied Biosystems' Assay on Demand service or designed as previously described in sections 3.2 and 5.2. Details of all primer/probe sets are given in these preceding chapters. Analysis of data and statistical analysis was performed as described in section 2.5, using one-way analysis of variance. LNG-IUS samples were run alongside the samples described in section 3.2 (normal endometrium from across the menstrual cycle) and analysed relative to the same comparator, a proliferative endometrial sample.

6.2.4 Immunohistochemistry

Immunohistochemistry was performed with specific antibodies against 11βHSD-1 and -2, GR, MR, 3βHSD and AKR1C3 (17βHSD-5) and standard methods as described in section 2.6 using biotin-conjugated secondary antibodies and ABC-Elite avidin biotin peroxidase complex (Vector). Immunoreactivity was detected using DAB (Dako).

MR immunostaining was performed using a Bond-X automated immunostaining robot as described in section 2.6.

All the antibodies used, specific conditions and controls were described previously in sections 3.2 and 5.2. Semi-quantitative visual analysis and statistics were performed as described in section 2.6 using the Kruskall-Wallis test.

6.3 Results

6.3.1 11βHSD mRNA expression

11βHSD-1 mRNA was expressed in pseudo-decidualised endometrium at greater levels than in proliferative and secretory endometrium, but lower than menstrual endometrium. The level of mRNA expression in pseudo-decidualised endometrium was similar to that in first trimester decidua. These data were not found to be statistically significant.

11βHSD-2 mRNA was expressed in pseudo-decidualised endometrium. Levels did not differ significantly from any stage of the normal menstrual cycle. Expression was greater than in first trimester decidua, although again this increase in expression was not statistically significant.

These data are shown in Figure 6.1.

6.3.2 11βHSD-1 protein expression

Expression of 11βHSD-1 protein was very low in LNG-exposed endometrium, this observation did not differ significantly compared to normal endometrium or first trimester decidua. 11βHSD-1 was mainly expressed in the cytoplasm of the glandular and surface epithelia. Immunoreactivity was not seen in all samples. These data are

shown in Figure 6.2. Charts showing immunoscores are included in Figure A7 of Appendix 1.

6.3.3 11βHSD-2 protein expression

11βHSD-2 protein was localised predominantly to the cytoplasm of the epithelia and stroma of pseudo-decidualised endometrium. There was an upregulation of expression in the stromal compartment after 3 months LNG-IUS usage and no further increase over time, although this was not found to be statistically significant. There was no significant difference in expression in either the surface or glandular epithelia. Expression in the vasculature was very low, and variable. It did not differ significantly over time. These data are shown in Figure 6.3. Charts showing immunoscores are included in Figure A8 of Appendix 1.

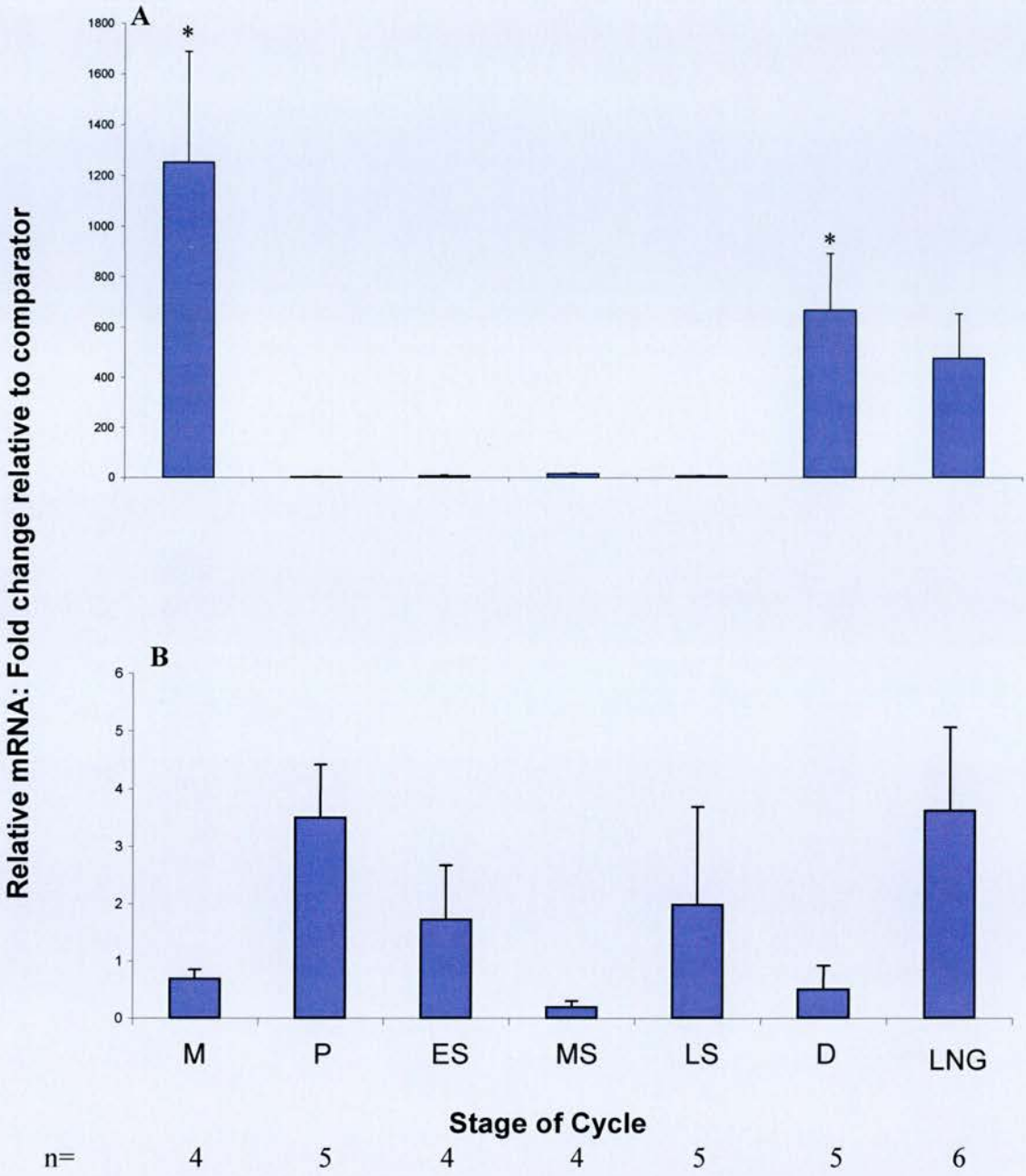


Figure 6.1 Expression of 11βHSD-1 and -2 mRNA across the menstrual cycle, in first-trimester decidua, and pseudo-decidualised endometrium, mean ± SEM. A=11βHSD-1; B=11βHSD-2. M = menstrual, P = proliferative, ES = early secretory, MS = mid secretory, LS = late secretory, D = decidua, LNG=LNG exposed (>1 year). The comparator was a proliferative endometrium sample. *$p<0.05$ compared to all other stages of the menstrual cycle.

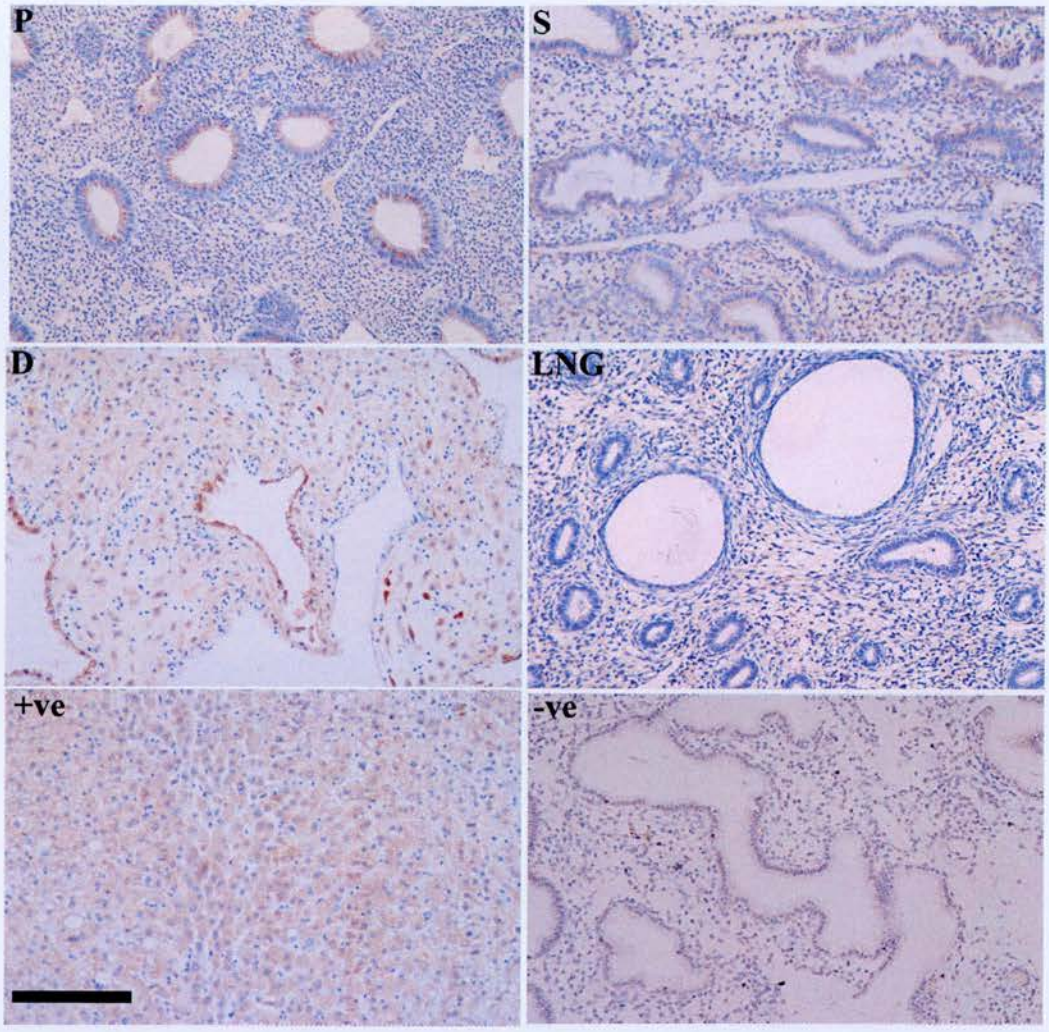


Figure 6.2 Immunolocalisation of 11βHSD-1 protein in normal and pseudo-decidualised endometrium (>1 year post-insertion of LNG-IUS). P=Proliferative endometrium, S=secretory endometrium, D=first trimester decidua, LNG=pseudo-decidualised endometrium, +ve= positive control, human liver, -ve=negative control, primary antibody substituted with matched IgG. Scale bar=10 microns.

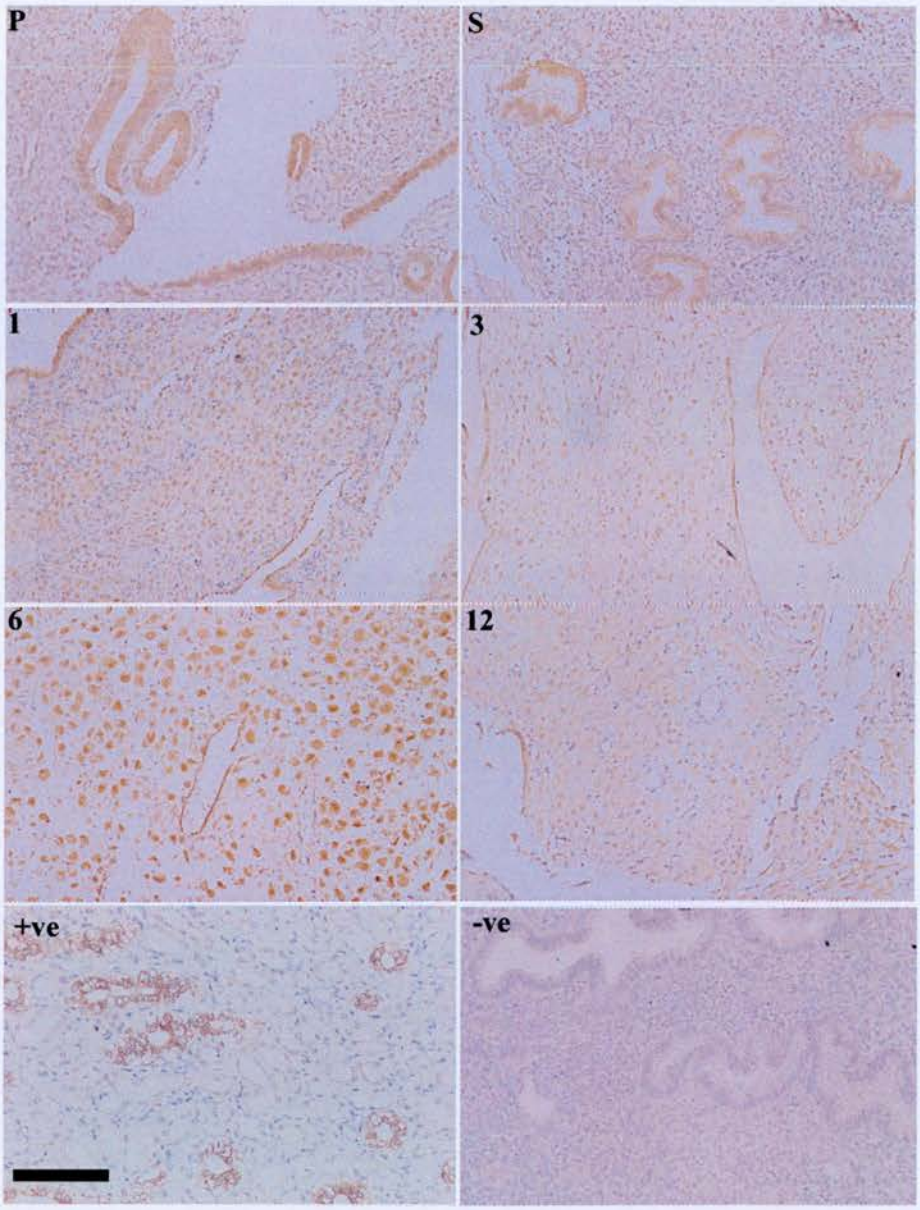


Figure 6.3 Immunolocalisation of 11βHSD-2 protein expression in normal and pseudo-decidualised endometrium over a period of 12 months. P=proliferative endometrium prior to insertion, S=secretory endometrium prior to insertion (samples collected depending on stage of cycle at time of insertion), 1=1 month post insertion, 3=3 months post insertion, 6=6 months post insertion, 12=12 months post insertion, +ve= positive control, human kidney, -ve=negative control, primary antibody substituted for matched IgG.. Sections shown are representative of each group and did not necessarily come from the same patient. Bottom right inset shows negative control, primary antibody replaced with matched IgG. Scale bar=10 microns.

6.3.4 GR and MR mRNA expression

GR mRNA was expressed in pseudo-decidualised endometrium. Levels did not differ significantly from any stage of the normal cycle. Expression in pseudo-decidualised endometrium was greater than in first trimester decidua although this was not statistically significant.

MR mRNA was expressed in pseudo-decidualised endometrium at broadly similar levels to all stages of the normal menstrual cycle and first trimester decidua.

These data are shown in Figure 6.4

6.3.5 GR protein expression

GR protein was also expressed in the nuclei of all cellular compartments of pseudo-decidualised endometrium. There was no significant difference in expression between pre- and post-insertion samples, or over time in the stroma, vessels, or surface epithelia. Expression was greatest in the glandular epithelia and was upregulated one month after insertion of LNG-IUS compared to pre-insertion (proliferative and secretory phase). There was a small decline in expression after 3 months and thereafter expression was constant. Again these data were not found to be statistically significant, and are illustrated in Figure 6.5. Charts showing immunoscores are included in Figure A9 of Appendix 1.

6.3.6 MR protein expression

MR protein was expressed most strongly in the glandular and surface epithelia of LNG-exposed endometrium. There was some expression in the stroma and little in the vasculature. Expression did not differ significantly from normal endometrium at any stage of the cycle or first trimester decidua. Expression was predominantly stromal. These data are shown in Figure 6.6. Charts showing immunoscores are included in Figure A10 of Appendix 1.

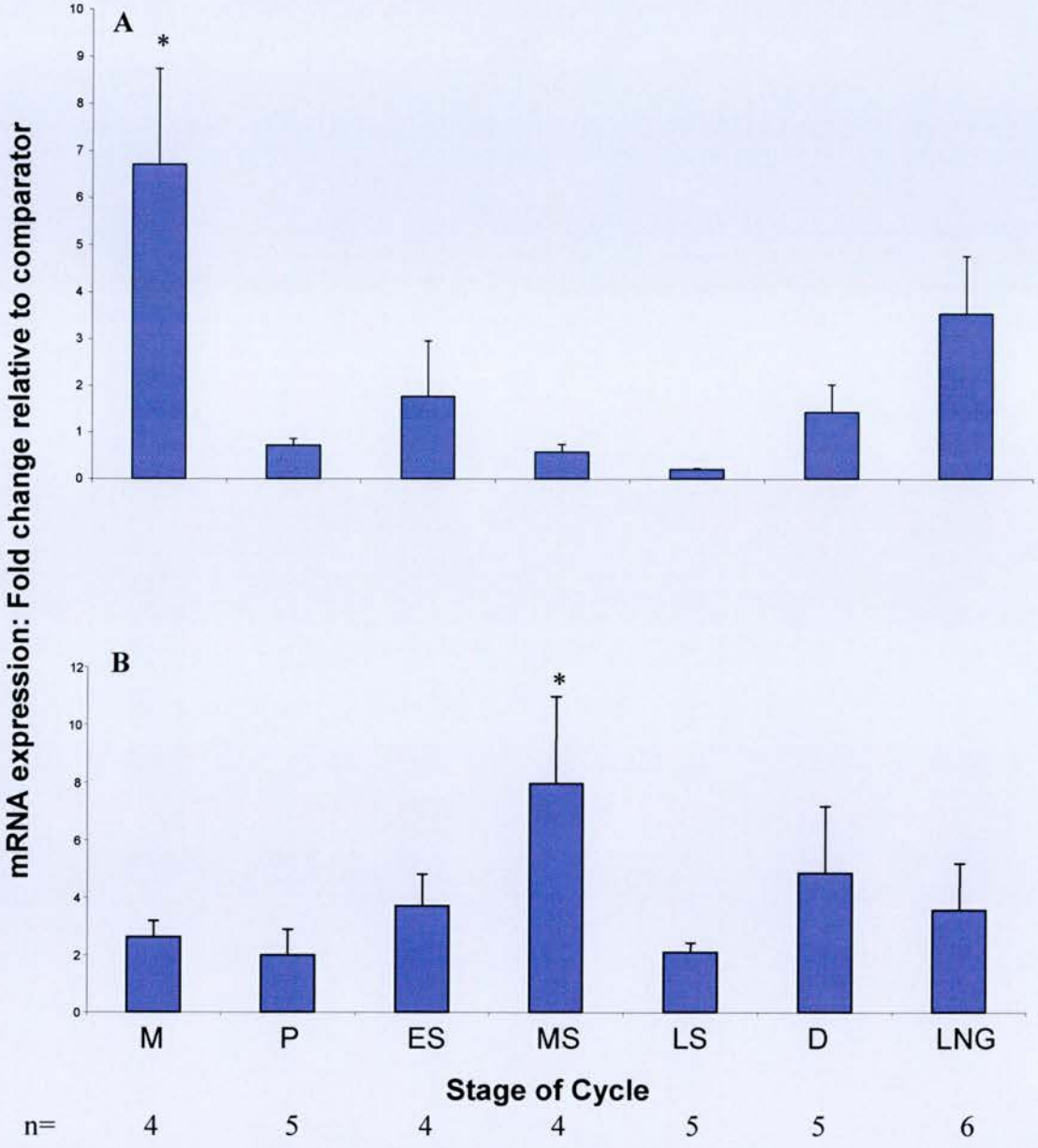


Figure 6.4 Expression of GR and MR mRNA across the menstrual cycle, in first-trimester decidua, and pseudo-decidualised endometrium, mean ± SEM. A=GR, B=MR. M = menstrual, P = proliferative, ES = early secretory, MS = mid secretory, LS = late secretory, D = decidua, LNG=LNG exposed (>1 year). The comparator was a proliferative endometrium sample. *$p<0.05$ compared to all other stages of the cycle.

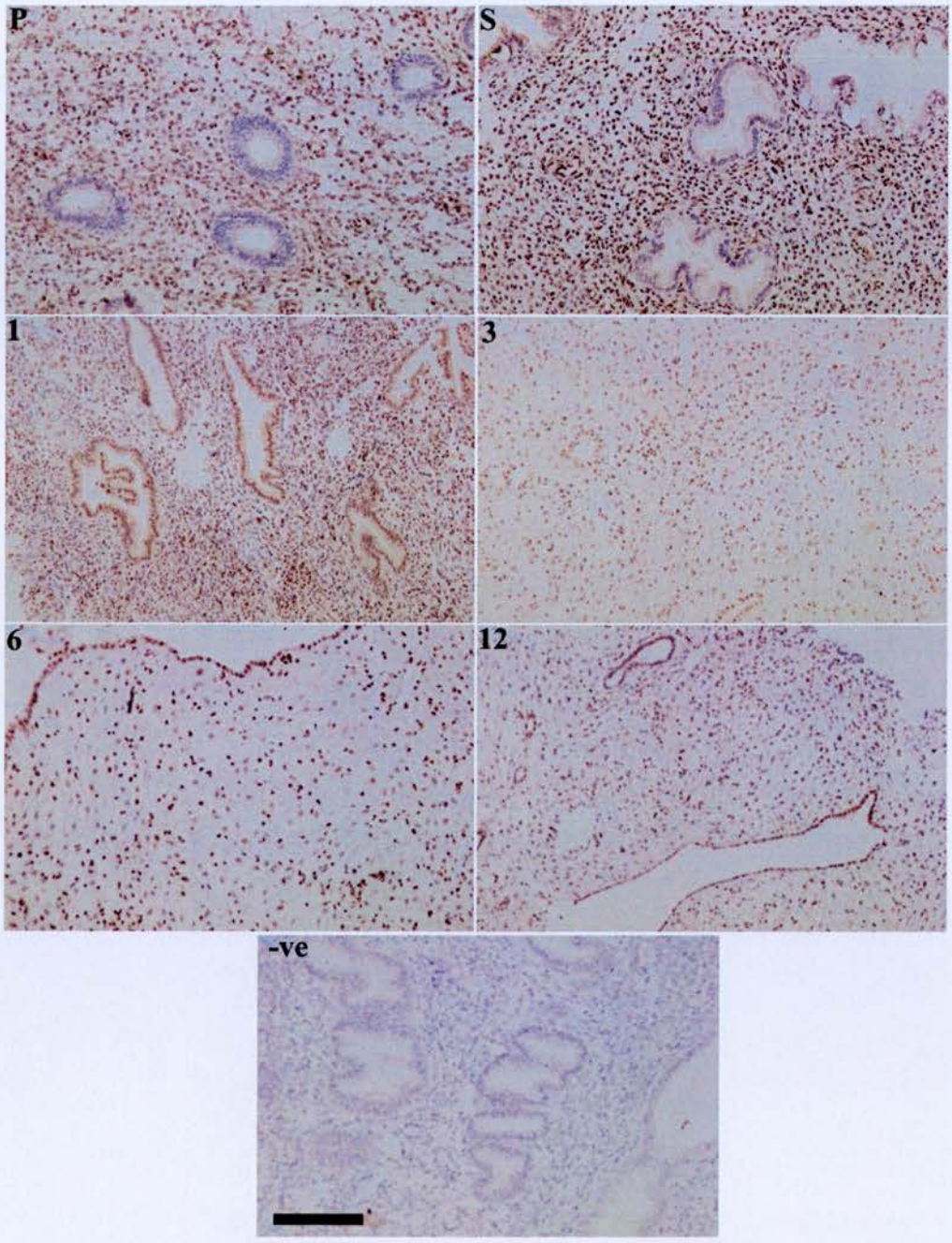


Figure 6.5 Immunolocalisation of GR protein expression in endometrium exposed to LNG over a period of 12 months. P=proliferative endometrium prior to insertion, S=secretory endometrium prior to insertion, 1=1 month post insertion, 3=3 months post insertion, 6=6 months post insertion, 12=12 months post insertion, -ve=negative control, primary antibody substituted for matched IgG.. Scale bar=10 microns.

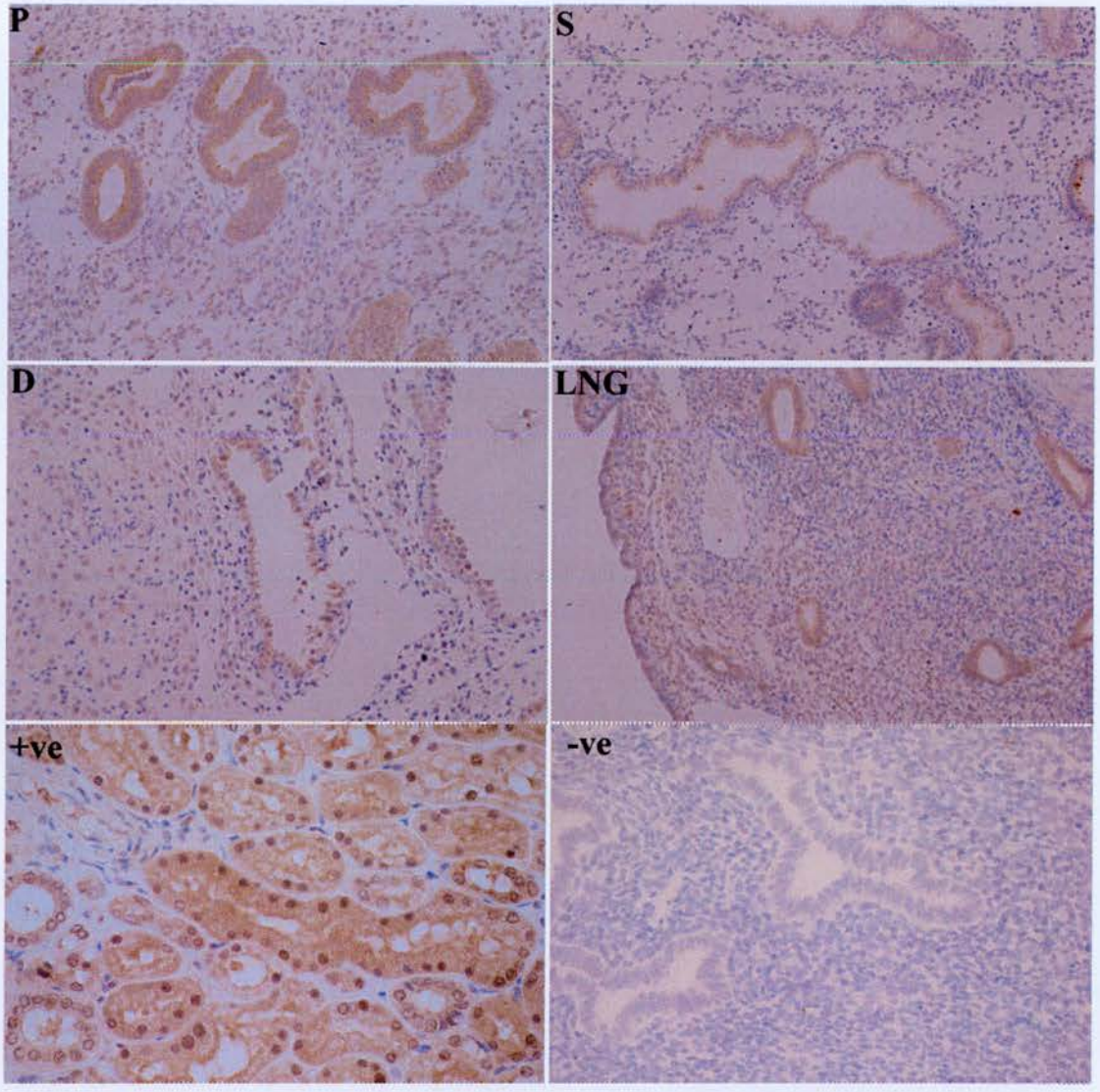


Figure 6.6 Immunolocalisation of MR protein in normal and pseudo-decidualised endometrium. P=Proliferative endometrium, S=secretory endometrium, D=first trimester decidua, LNG=pseudo-decidualised endometrium (>1 year post-insertion), +ve= positive control, human kidney, -ve=negative control, primary antibody substituted for matched IgG. Scale bar=10 microns

6.3.7 3βHSD mRNA expression

3βHSD-1 was expressed in pseudo-decidualised endometrium at greater levels than in first trimester decidua. There was no detectable expression in normal endometrium. These data are shown in Figure 6.7.

3βHSD-2 mRNA was not detectable in pseudo-decidualised endometrium, as was also the case in normal endometrium and first trimester decidua.

6.3.8 3βHSD protein expression

3βHSD protein was expressed in the stroma at low levels in all cellular compartments. There was no difference in expression between pre- and post-insertion samples, or in any of the timed post-insertion biopsies in the stroma, surface epithelia and vessels. Greatest expression was seen in the glandular epithelia,with a non-significant increase 1 month after LNG-IUS insertion although expression was still at low levels. There was a small decline in expression after 3 months post LNG-IUS insertion, although again this was a non-significant observation. These data are shown in Figure 6.8. Charts showing immunoscores are included in Figure A11 of Appendix 1.

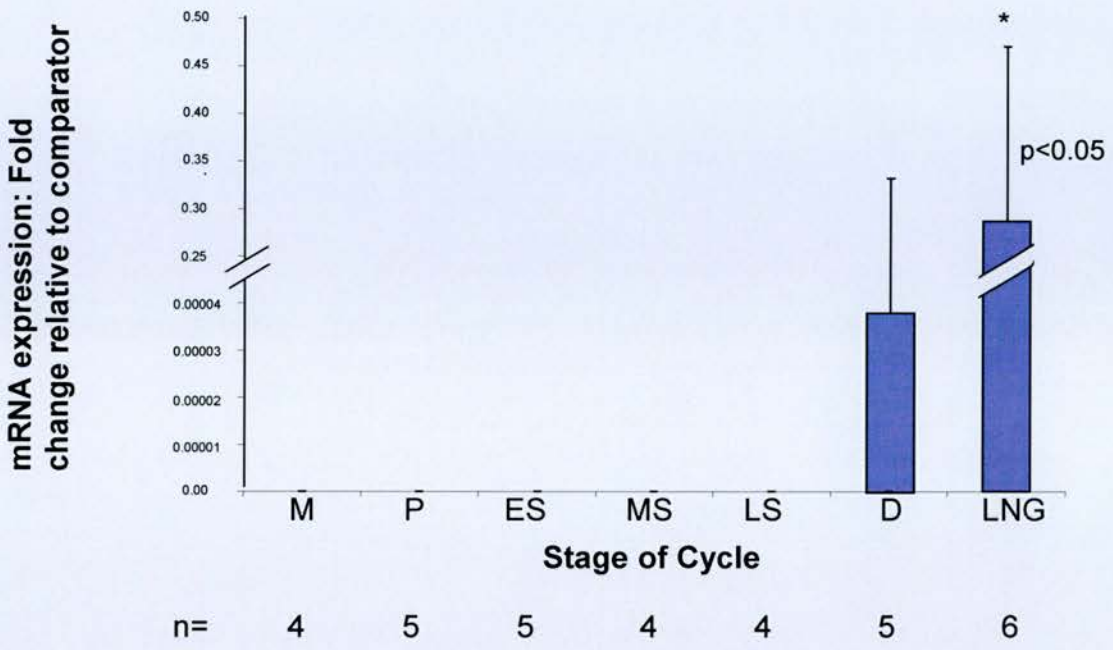


Figure 6.7 Expression of 3βHSD-1 mRNA across the menstrual cycle, in first-trimester decidua and pseudo-decidualised endometrium, mean ± SEM. M = menstrual, P = proliferative, ES = early secretory, MS = mid secretory, LS = late secretory, D = decidua, LNG=LNG exposed (>12 months). The comparator was a first trimester decidua sample, used as this group was the only "normal" group to show any 3βHSD-1 expression . *$p<0.05$ compared to first trimester decidua. Statistical analysis was not possible against normal endometrial samples as 3βHSD-1 was not detectable. Axis is split to allow both bars to be shown on the same chart.

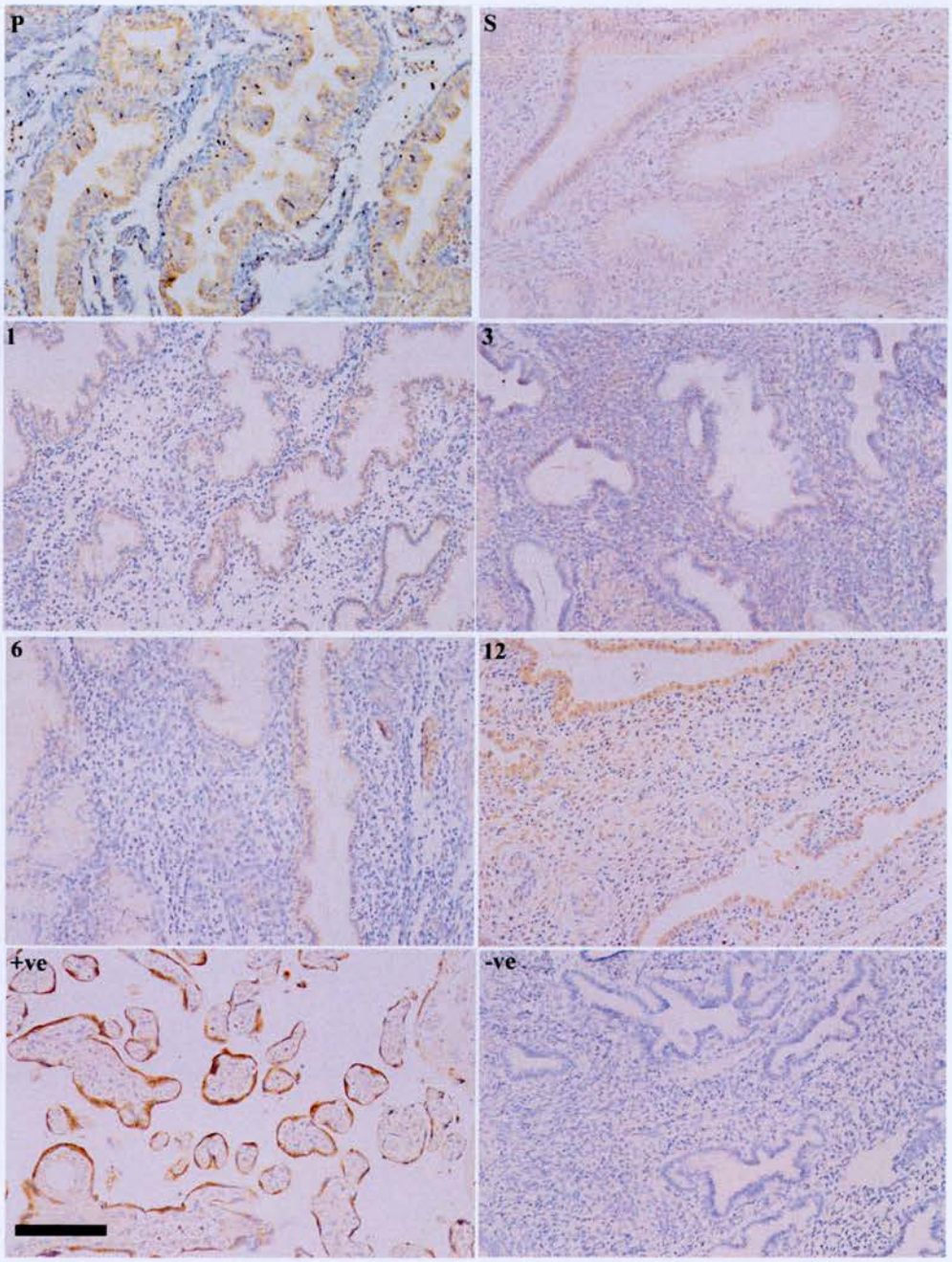


Figure 6.8 Immunolocalisation of 3βHSD protein expression in normal and pseudo-decidualised endometrium over a period of 12 months. P=proliferative endometrium prior to insertion, S=secretory endometrium prior to insertion, 1=1 month post insertion, 3=3 months post insertion, 6=6 months post insertion, 12=12 months post insertion, +ve= positive control, human placenta, -ve=negative control, pre-immune serum. Scale bar=10 microns

6.3.9 AKR1C1-1C4 mRNA expression

AKR1C1 mRNA was expressed in pseudo-decidualised endometrium. Expression was greater than that seen at any stage of the cycle in normal endometrium, or in first trimester decidua.

AKR1C2 mRNA expression in LNG-exposed endometrium was at similar levels to first trimester decidua. Expression did not differ significantly from any stage of the normal cycle.

Expression of AKR1C3 (17βHSD-5) mRNA in LNG-exposed endometrium was significantly lower than that in early secretory endometrium. Expression was not significantly different to any other stage of the menstrual cycle, or first trimester decidua.

Data are shown in Figure 6.9.

AKR1C4 mRNA was not expressed in pseudo-decidualised endometrium.

6.3.10 AKR1C3 (17βHSD-5) protein

AKR1C3 (17βHSD-5) protein was localised to the cytoplasm of the glandular and surface epithelia and the endothelial cells of the vasculature of pseudo-decidualised endometrium. Expression was high although there was a small decline in expression in first trimester decidua and pseudo-decidualised endometrium in comparison to normal endometrium, but this was not statistically significant. These data are illustrated in Figure 6.10. Charts showing immunoscores are included in Figure A12 of Appendix 1.

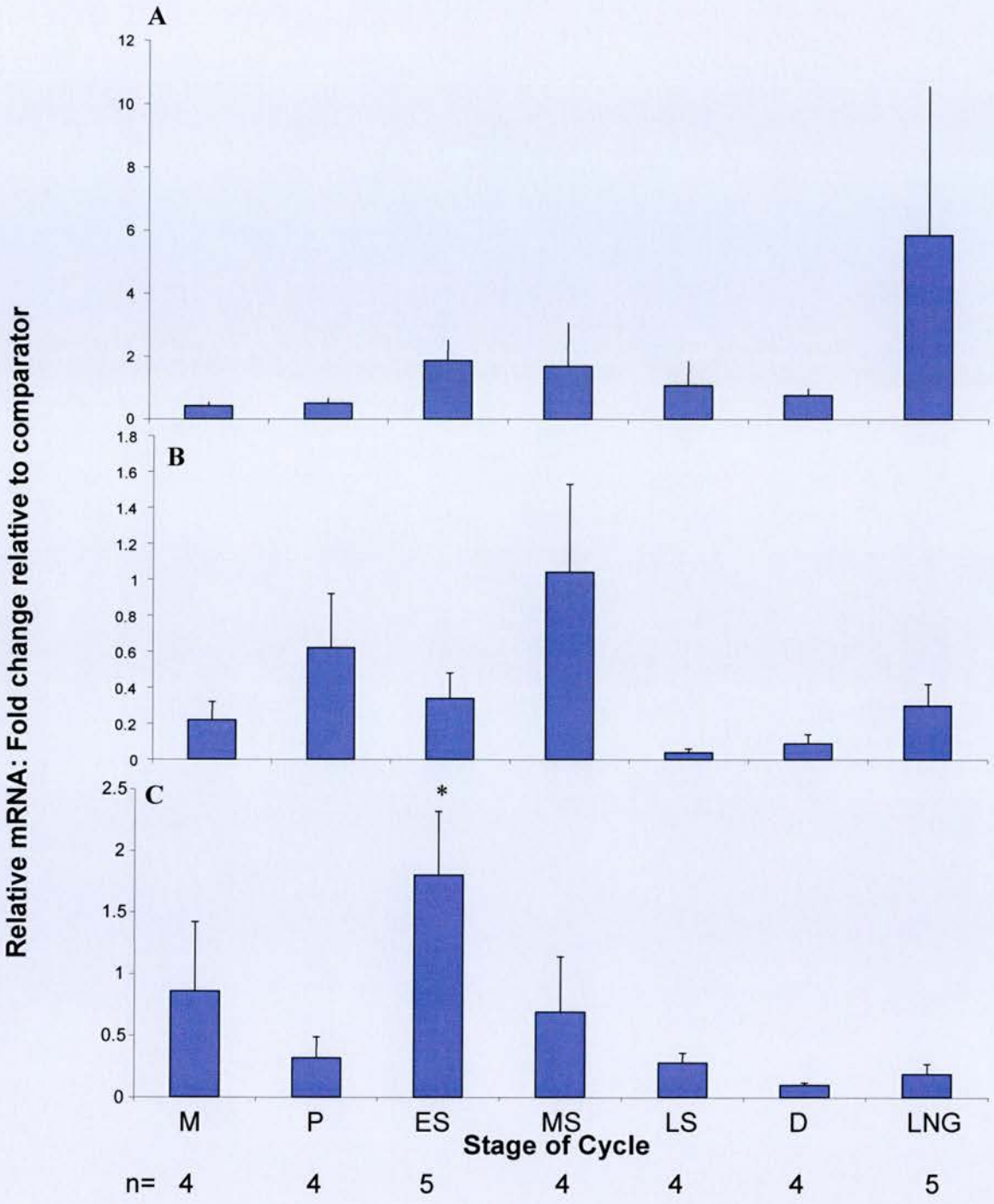


Figure 6.9 Expression of AKR1C1-1C3 mRNA across the menstrual cycle, in first-trimester decidua and pseudo-decidualised endometrium, mean ± SEM M = menstrual, P = proliferative, ES = early secretory, MS = mid secretory, LS = late secretory, D = decidua, LNG=LNG exposed. A=AKR1C1, B=AKR1C2, C=AKR1C3 (17HSD-5). The comparator was a proliferative endometrium sample.*$p<0.05$ compared to all other stages of the menstrual cycle.

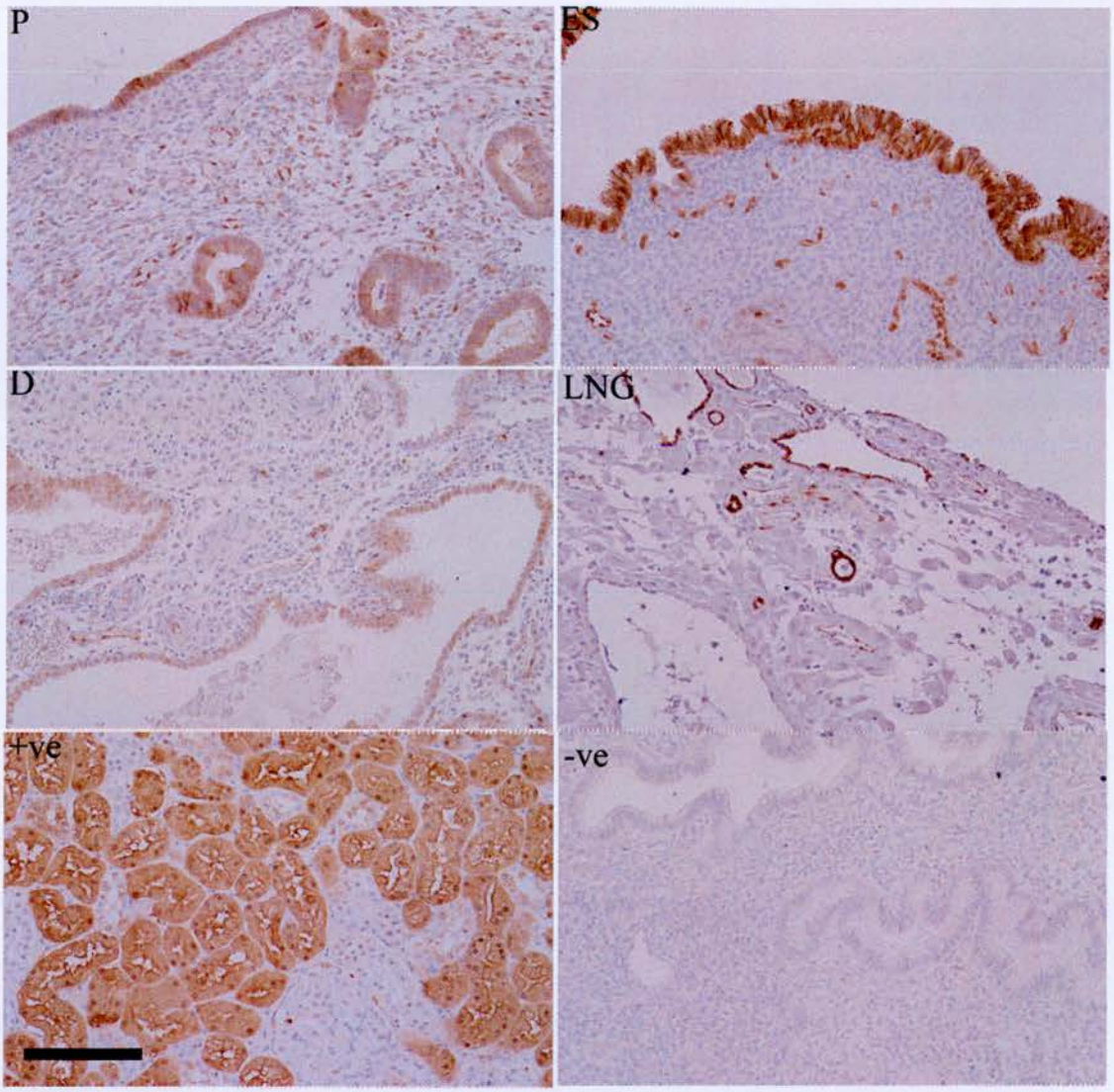


Figure 6.10 Immunolocalisation of AKR1C3 (17βHSD-5) protein expression in normal pseudo-decidualised endometrium. P=Proliferative endometrium, S=secretory endometrium, D=first trimester decidua, LNG=pseudo-decidualised endometrium, +ve= positive control, kidney, -ve=negative control, primary antibody substituted for matched IgG. Scale bar=10 microns.

6.4 Discussion

The 11βHSD enzymes create a cortisol-cortisone "shuttle" whereby 11βHSD-1 converts inactive cortisone to the active glucocorticoid cortisol and 11βHSD-2 performs the reverse reaction *in vivo*.

11βHSD-1 mRNA was upregulated in LNG-exposed endometrium compared with proliferative and secretory endometrium. Expression levels were similar to those in first-trimester decidua, suggesting a similar role for 11βHSD-1 in LNG-exposed endometrium as in first trimester decidua. Further investigation may allow 11βHSD-1 to be defined as a marker of both decidualisation and pseudo-decidualisation. However 11βHSD-1 protein was only expressed at low levels in pseudo-decidualised endometrium and was not seen in all samples. It seems that the mRNA may not be translated. As most of the subjects in this group attended clinic due to bleeding problems while using the LNG-IUS, the precise aetiology of erratic and unsubstantiated bleeding patterns in these patients is not known although lots of evidence strongly point to problems of vascular integrity. The upregulation of the mRNA suggests there is some inflammatory response occurring in pseudo-decidualised endometrium, as is seen in first trimester decidua. Perhaps patients who did not experience breakthrough bleeding would have been appropriate controls for this study, however these patients did not return to clinic for follow-up biopsies other than in the timed study. As there were insufficient

samples in this set to conduct all the analyses it was decided to use pre-insertion endometrium as controls in all experiments.

11βHSD-2 mRNA expression was greater in pseudo-decidualised endometrium than in first trimester decidua, although this was not statistically significant. Expression did not differ significantly when compared to any stage of the normal cycle either. This small increase compared to first trimester decidua could be in order to balance the increase in 11βHSD-1 expression and thus modulate the potential prolonged inflammatory response. 11βHSD-2 protein was studied at timed intervals after insertion of an LNG-IUS. A small upregulation was seen in expression in the stroma after 6 months of usage. This is also observed in the first trimester of pregnancy where expression is seen in the stroma as well as the epithelia. There was no further change in expression over time. This corresponds with the mRNA data.

The ratio of 11βHSD-1 and -2 expression is also important in modulating the local availability of cortisol. In the present study, the increase in expression of 11βHSD-1 mRNA is greater than the non-significant upregulation of 11βHSD-2 mRNA, thus favouring 11βHSD-1 action, leading to the potential for increased cortisol production. Small et al. (2005) showed 11βHSD-1 to amplify the angiostatic effect of glucocorticoids in a rodent model, and studies in our laboratory have followed up these observations in human endometrium, and found cortisol to also inhibit angiogenesis in the human endometrium.

Previous studies have shown 11βHSD-1 and -2 to be expressed in the blood vessel wall in humans (Hadoke et al., 2001; Cai et al., 2001; Christy et al., 2003). Expression of both 11βHSD-1 and -2 was detected in the vasculature in the present study, although at low and variable levels in both non-pregnant and pseudo-decidualised endometrium. However there was no significant difference between normal and LNG-exposed endometrium, thus we cannot draw any firm conclusions as to the role of 11βHSD-1 and -2 in the aberrant bleeding often experienced by users of the LNG-IUS.

It has been proposed that breakthrough bleeding may be a result of MMP activation (Hickey and Fraser, 2000), and glucocorticoids are thought to be regulators of MMPs. Thus alterations in 11βHSD expression, and consequently glucocorticoid availability, could have an indirect effect in pseudo-decidualised endometrium and contribute to breakthrough bleeding.

The corresponding cognate receptors for cortisol, GR and MR, were also studied. GR mRNA expression in LNG-treated endometrium was not significantly different to normal endometrium or first trimester decidua, although there was a small increase in comparison to the first trimester decidua. This corresponds to the slightly increased 11βHSD-1 mRNA, which can allow increased cortisol availability and elicit a glucocorticoid response via the GR. GR protein was also studied in timed biopsies after insertion of LNG-IUS. Expression was seen in both glandular and stromal compartments, as is the case in first trimester decidua. There was a small non-significant

increase in glandular epithelia one month after insertion, which again corresponds to the small increase seen at the mRNA level.

GR protein was also expressed consistently in the nuclei of endothelial cells of both non-pregnant and pregnant endometrium and pseudo-decidualised endometrium following LNG-IUS use. GR has been previously reported to be expressed in the endothelial cells of both endometrium and decidua (Bamberger et al., 2001; Henderson et al., 2003). This suggests that breakthrough bleeding associated with LNG-IUS useage is unlikely to be due to altered GR expression in the endothelium. However, this study did not take into account expression levels at different tissue locations. It has been previously reported that breakthrough bleeding occurs from more superficial, dilated vessels (Hickey et al., 1996) so perhaps an alteration would be seen in GR or 11βHSD-1 expression if these superficial vessels were compared to those deeper into the tissue.

MR mRNA was expressed in LNG-exposed endometrium and levels did not significantly differ from any stage of the cycle or first trimester decidua. This was mirrored by the protein expression which was localised to the epithelia. This suggests any effect of cortisol on LNG-exposed endometrium is likely acting through the increased levels of GR. The small increase in 11βHSD-2 seen will add further protection to MR and direct cortisol to bind to the GR. This also suggests there is no additional requirement for mineralocorticoid action in response to continual exposure to progestogen.

3βHSD-1 and -2 are isoforms of an enzyme that produces progesterone from pregnenolone and also has the ability to metabolise a number of androgens.

3βHSD-1 mRNA was upregulated in both pseudo-decidualised endometrium and first trimester decidua compared to normal endometrium. There was a further significant increase in expression in pseudo-decidualised endometrium compared to first trimester decidua, although expression levels remained very low. This could be due to progestogen (LNG) acting in a feed-forward manner to further increase the level of progesterone available. It has been shown that that cortisol acts on 11βHSD-1 in this manner (Yong et al., 2002; Rae et al., 2004a). 3βHSD-2 mRNA was not detectable in pseudo-decidualised endometrium. 3βHSD protein (studied using an antibody that detects both isoforms, as described in section 5.2) was expressed in the glands and some expression was seen in the vasculature of LNG-exposed endometrium. This suggests 3βHSD and perhaps the corresponding progesterone increase has a role in the regulation of bleeding. However, as progesterone is withdrawn in the absence of pregnancy and induces bleeding, it may be that 3βHSD is acting on androgens to cause irregular bleeding, The precise role of androgens in the endometrium has not yet been well defined, however they have been proposed to have an inhibitory effect on the female reproductive tract, including endometrial atrophy (Miller et al., 1986; Futterweit & Deligdisch, 1986; Slayden et al., 2001). Okon et al. (1998) showed exogenous androgen to block estrogen action in the endometrium of ovariectomised macaques. Androgens act via the AR, the expression patterns of which are the subject of some conflict between researchers, perhaps due to differences in immunohistochemical methods. Horie et al.

(1992) and Ito et al. (2002) have found AR to be expressed in both glandular and stromal cells of the endometrium, whereas other authors have reported stromal localisation of AR (Maia et al., 2001; Mertens et al., 2001; Slayden et al., 2001). Slayden et al. (2001) have also reported AR to be expressed in the endometrium in a similar manner to ER and PR; greatest expression in the estrogen-dominated proliferative phase and downregulated in the secretory phase. AR is also expressed in decidual stromal cells and endothelial cells (Milne et al., 2005). In endometrium exposed to levonorgestrel, there is a significant decrease in AR expression in the stroma, to levels similar to those seen in the secretory phase (Burton et al., 2003). It has been shown previously that PR is downregulated in pseudo-decidualised endometrium (Critchley et al., 1998), and consequently the endometrium is less responsive to progesterone-mediated events. This may be a response to counter the continual exposure of the endometrium to progestogen and thus lends weight to the theory that 3βHSD is acting on androgen metabolism in this tissue, rather than progesterone formation.

The predominant activity of AKR1C1 is to reduce progesterone to 20α-hydroxyprogesterone, a less potent ligand. AKR1C1 mRNA expression was upregulated in relation to normal endometrium and first trimester decidua. Thus AKR1C1 may act in balance with 3βHSD to modulate the availability of active progesterone. AKR1C1 may also be responsible for bringing about a progesterone withdrawal response and inducing bleeding in users of the LNG-IUS. Unfortunately it was not possible to study this enzyme in timed biopsies following insertion, to investigate whether AKR1C1

expression is downregulated after a period of LNG-IUS use, corresponding to the reduction in bleeding experienced in many users in time.

AKR1C2 acts to reduce potent androgens to less potent forms. AKR1C2 mRNA expression in pseudo-decidualised endometrium was similar to that in both normal endometrium and first trimester decidua. As there is no difference in AKR1C2 expression in pseudo-decidualised endometrium, it is unlikely that AKR1C2 plays a major role in the problem of breakthrough bleeding.

AKR1C3 (17βHSD-5) acts to produce active androgens and estrogens from less potent forms. AKR1C3 (17βHSD-5) mRNA was significantly reduced in pseudo-decidualised endometrium compared to early secretory endometrium, but did not differ significantly from the other stages of the cycle. Expression was low in both first trimester decidua and LNG-exposed endometrium. In first trimester decidua, estrogen levels are low, as would be expected in pseudo-decidualised endometrium. Thus, low expression of AKR1C3 will help to maintain these low estrogen levels. However AKR1C3 protein was highly expressed in LNG-exposed endometrium at levels not significantly different to those in normal endometrium or first trimester decidua, although there was a small non-significant decline in both first trimester decidua and pseudo-decidualised endometrium. Protein expression was localised to the epithelia and endothelial cells of the vessels. Strong expression in the vasculature implies a role for AKR1C3 in the regulation of bleeding. Reduced levels ofAKR1C3 (17βHSD-5) would lead to reduced local amounts of both estrogen and testosterone. The cognate receptors for these steroids, ER and AR

have also been shown to be downregulated in pseudo-decidualised endometrium (Critchley et al., 1998; Burton et al., 2003).

AKR1C4 controls the expression of 5α/5β tetrahydrosteroid. It was not expected to detect AKR1C4 in pseudo-decidualised endometrium, as this enzyme has been previously reported to be liver specific, and was also shown in section 5.3 of this thesis not to be detectable in normal human endometrium, and this was indeed shown to be the case. AKR1C4 expression is not induced by continued exposure to progestogen.

Concluding remarks

Table 6.1 summarises the expression patterns of the enzymes studied in endometrium exposed to LNG, and the effect on steroid hormone activity.

Table 6.1 Summary of enzyme expression.

Enzyme	mRNA expression	Protein Expression	Predominant Product	Activity
11βHSD-1	↑	↓	Cortisol	↑
11βHSD-2	↑	↔	Cortisone	↓
3βHSD-1	↑	n/a	Progesterone, testosterone	↑
3βHSD-2	-	↑	Progesterone, testosterone	↑
AKR1C1	↑	n/a	20αOHP	↓
AKR1C2	↔	n/a	Androstendiol/testosterone	↔
AKR1C3	↓	↔	Androstenedione, estrone	↓

Many of the steroid metabolising enzymes and receptors studied here are expressed in similar patterns in LNG-exposed endometrium and first trimester decidua, consistent

with observations that LNG induces morphological pseudo-decidualisation. There are, however, some differences, notably in 3βHSD expression. It would be desirable to further understand the mechanisms relating to breakthrough bleeding, and a possible method for this would be to study these and other enzymes and receptors in users of the LNG-IUS who have had breakthrough bleeding, in comparison with those who have not.

Chapter 7:

Effect of exogenous steroid manipulation (GnRH antagonist treatment) on sex steroid pre-receptor signalling

7.1 Introduction

Gonadotrophin-releasing hormone (GnRH) antagonists, of which Cetrorelix is an example, have recently become widely used in treatment of sub-fertility. During in-vitro fertilisation (IVF) and intra-cytoplasmic sperm injection (ICSI), treatment protocols, it is desirable to prevent a premature luteinising hormone (LH) surge (Olivennes et al., 2000; Albano et al., 2000). GnRH antagonists suppress LH and consequently eliminate the possibility of a premature LH surge (Albano et al., 2000). An advantage of using GnRH antagonists for this purpose instead of the previously used GnRH agonists is that there are no menopausal side effects caused by the estrogen deprivation associated with GnRH agonist treatment. The amount of gonadotrophin required is also reduced with GnRH antagonist treatment.

There are concerns however about a potential reduction in pregnancy rates when GnRH antagonists are used in IVF/ICSI treatments, compared with the use of GnRH agonists (Al-Inany et al., 2002). This area is controversial as some studies have shown both methods to produce similar pregnancy rates (Ludwig et al., 2001). It is however currently unclear how these treatments affect the reproductive system. In particular, effects of GnRH antagonists on the endometrium are not well described. There is however evidence of premature advancement of the endometrium at the time of oocyte retrieval following use of GnRH antagonists and recombinant follicle stimulating

hormone (recFSH) (Kolibianakis et al., 2002; 2003). This has been associated with failure of implantation.

The mechanisms involved in implantation have received considerable attention, however they have not yet been fully elucidated. Implantation involves an interaction between the endometrium and embryo, mediated by a number of factors including growth factors (Bausero et al., 1998), hormones, adhesion molecules (Horne et al., 2000; Lessey, 2002), extracellular matrix molecules (Jokimaa et al., 2000), cytokines and prostaglandins (Kelly et al., 2001). Sex steroids, in particular estrogens and progestogens are thought to play vital roles in endometrial development. These steroid ligands act via their cognate receptors (PR, ER, AR). The expression of these sex steroids and their cognate receptors is reviewed in section 1.2.4 of this thesis.

The ovarian stimulation that occurs during IVF and ICSI causes the levels of gonadotrophins to rise beyond physiological levels and this consequently has an effect on the expression of sex steroid receptors in the endometrium. A recent study in our laboratory (Vani et al., 2007) has shown that the local expression of sex steroid receptors, in particular ERα and PR, are perturbed. Thus it was desirable to investigate the effect of this GnRH antagonist administration on expression of the enzymes that control availability of ligands for ER and PR – estrogens and progesterone and DHEA to androstenedione. 3βHSD-1 and -2 are involved in the biosynthesis of all active steroids by catalysing one of the first steps, the conversion of pregnenolone to progesterone. 17βHSD-2 and -5 (AKR1C3) act to modulate the availability of androgens and

estrogens. The key reactions of these enzymes have been summarised previously in Table 5.1. Thus, expression of 17βHSD-2 and AKR1C3 (17βHSD-5) and 3βHSD-1 and -2 were examined in endometrium exposed to GnRH antagonist treatment.

The aims of this chapter were to investigate the effects of GnRH antagonist treatment on endometrial expression of steroid metabolising enzymes, at both an mRNA and protein level. The following steroid metabolising enzymes were studied.

- 3βHSD-1
- 3βHSD-2
- 17βHSD-2
- AKR1C3 (17βHSD-5)

7.2 Materials and Methods

7.2.1 Subjects and tissue resource

Ethical approval and informed consent were obtained from all subjects. Five parous women who had come forward as oocyte donors were recruited as the study group. These women were donating eggs for use in fertility treatment by other patients and were not undergoing fertility treatment themselves.

Members of the study group underwent oocyte stimulation using the local assisted conception protocol as described by Thong et al. (2003). Briefly, recombinant FSH (Gonal-F, Serono, UK) treatment began on day 4 of their menstrual cycle to stimulate the ovaries. Cetrorelix (a GnRH antagonist) was administered daily from day 7 or 8, when follicles were measured to have reached 11mm and a transvaginal ultrasound was used to monitor the ovarian response. When 3 follicles with a diameter of at least 17mm were identified, hCG was administered to trigger oocyte maturation. Oocytes were retrieved 35-36 hours after hCG. Progesterone was then administered via a pessary every 12 hours from 2 days after oocyte retrieval until the endometrium was to be sampled. This is shown in schematic form in Figure 7.1. Endometrial biopsies were collected 8-10 days after hCG administration using a pipelle sampling device. These samples were used for immunohistochemistry.

Eight parous women with regular menses (25-35 days) were recruited as controls. These subjects were asked to provide a urinary sample on alternate days from day 10 of their cycle and LH levels in these samples were measured. Endometrial biopsies were performed 6-10 days after the peak of a urinary LH surge as shown in Figure 7.1. These women attended the gynaecology clinic for sterilisation or other complaints excluding menstrual problems or infertility. In all these women, the last menstrual period, endometrial histology and serum estradiol and progesterone levels were consistent with the mid secretory phase of the cycle, as the putative window of implantation falls within this phase.

Endometrial tissue was fixed in 4% paraformaldehyde then imbedded in paraffin for immunohistochemistry. Additional tissue was frozen at the point of collection in liquid nitrogen and stored at -70°C.

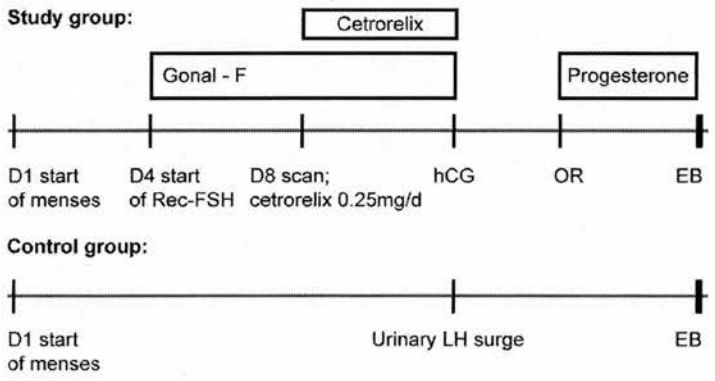


Figure 7.1 Diagrammatic representation of the Edinburgh Assisted Conception Protocol. D=day of cycle; hCG= human chorionic gonadotrophin administration; OR=oocyte retrieval; EB=endometrial biopsy (from Vani et al., 2007).

7.2.2 RNA extraction and reverse-transcriptase PCR

RNA extraction was performed using Trizol (Invitrogen) as described in section 2.3.3. The resulting RNA was treated with DNAse1, Amp grade 1U/μg RNA in DNase1 reaction buffer for 15 minutes at room temperature and the reaction stopped by the addition of EDTA. Reverse-transcriptase PCR was performed using standard methods as described in 2.4.

7.2.3 Taqman Quantitative Real-Time PCR

QRT-PCR was performed using standard methods as described in Chapter 2.5. Oligonucleotide forward and reverse primers and oligonucleotide Taqman probes were used to detect sequences of interest. The probes were commercially available from Applied Biosystems' Assay on Demand service or designed as previously described by Burton et al. (2003). The primer/probe sets from the Assay on Demand service were pre-validated and the sequences are not available. The reference numbers were given previously in Table 5.4. The sequences of the 17βHSD-2 primers and probes are as follows:

17βHSD-2 forward:	TGTCAGCAGCATGGGAGGA
17βHSD-2 reverse:	GGTCACAGCCGCCTTTGAT
17βHSD-2 probe:	CCCCAATGGAAAGGCTGGCATCTT

Analysis of data and statistical analysis was performed as described in section 2.5 and the data were log-transformed to allow statistical analysis on the small sample set and an unpaired t-test was performed.

7.2.4 Immunohistochemistry

Immunohistochemistry was performed using antibodies specific to 3βHSD and 17βHSD-5 and standard methods as described in 2.7 and using biotin-conjugated secondary antibodies and ABC-Elite avidin biotin peroxidase complex (Vector). Immunoreactivity was detected using DAB (Dako).

All the antibodies used were described previously in 5.2.4. The 3βHSD antibody, as described previously, detects both 3βHSD-1 and -2. A suitable antibody was not available for 17βHSD-2. Semi-quantitative visual analysis was conducted by two observers, scored blind on a 4-point scale. Data were then analysed using the Mann-Whitney test.

7.3 Results

7.3.1 3βHSD mRNA expression

Low levels of 3βHSD-1 mRNA were seen in mid-secretory control endometrium and these levels were further reduced in GnRH antagonist treated endometrium. This was found to be statistically significant (p=0.01). 3βHSD-2 mRNA was expressed at very low levels in mid-secretory endometrium, but was not detectable at all in GnRH-antagonist treated endometrium after 40 cycles of QRT-PCR. For comparison, 3βHSD-2 mRNA was detected in the control samples after 38 cycles of QRT-PCR. These data are shown in Figure 7.2.

7.3.2 3βHSD protein expression

3βHSD protein was predominantly expressed in the glandular and surface epithelia of both GnRH-antagonist treated endometrium and control endometrium. The protein was expressed at low levels and expression did not differ significantly between control and treated endometrium. These data are shown in Figure 7.3. Charts showing immunoscores are included in Figure A13 of Appendix 1.

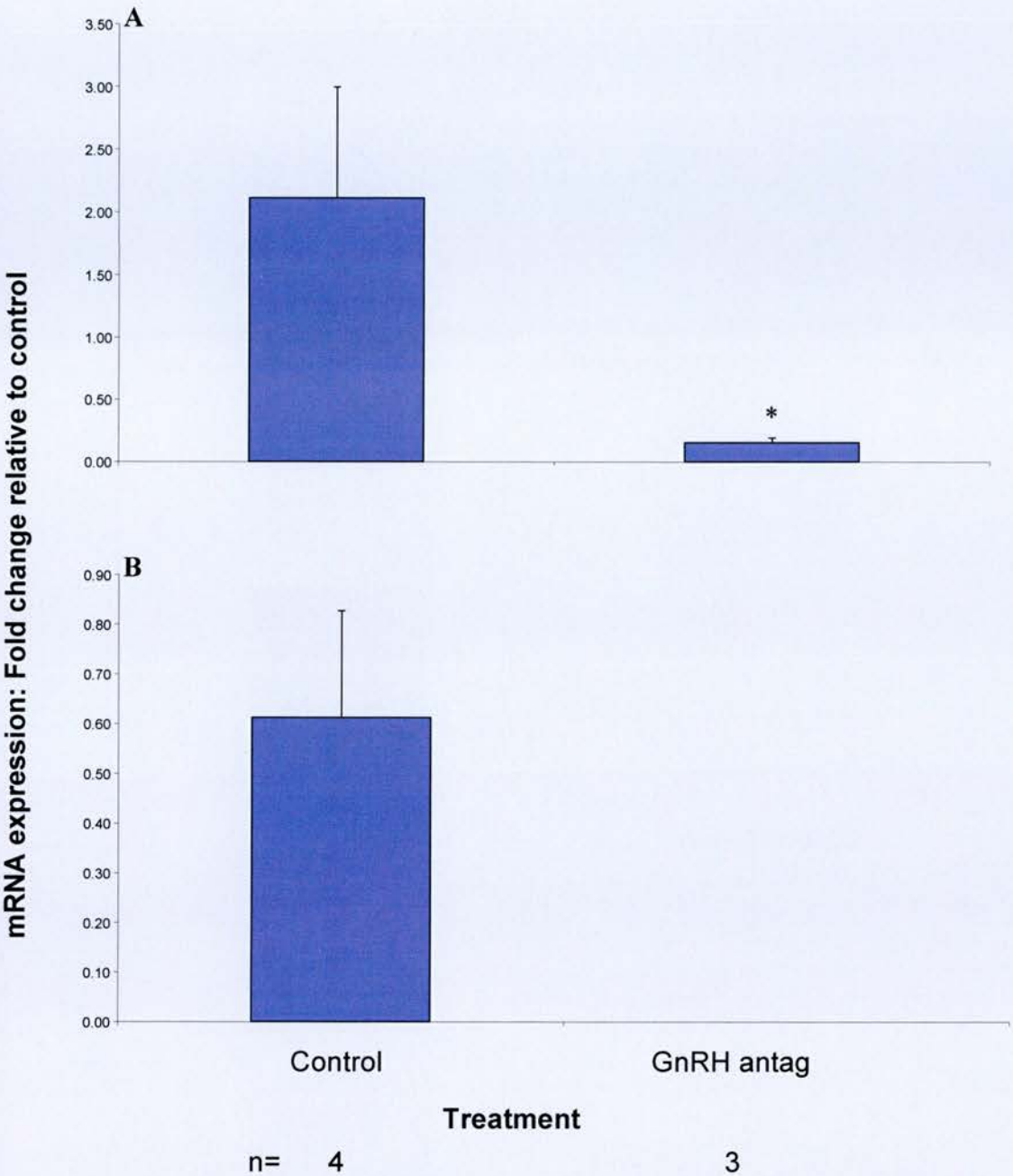


Figure 7.2 Expression of 3βHSD-1 and -2 mRNA in GnRH-antagonist treated endometrium compared to normal mid-secretory endometrium (control), mean ± SEM. The comparator was an untreated control sample. A=3βHSD-1, B=3βHSD-2. It was not possible to conduct statistical analysis on the 3βHSD-2 data as the mRNA was not detectable in one of the groups.

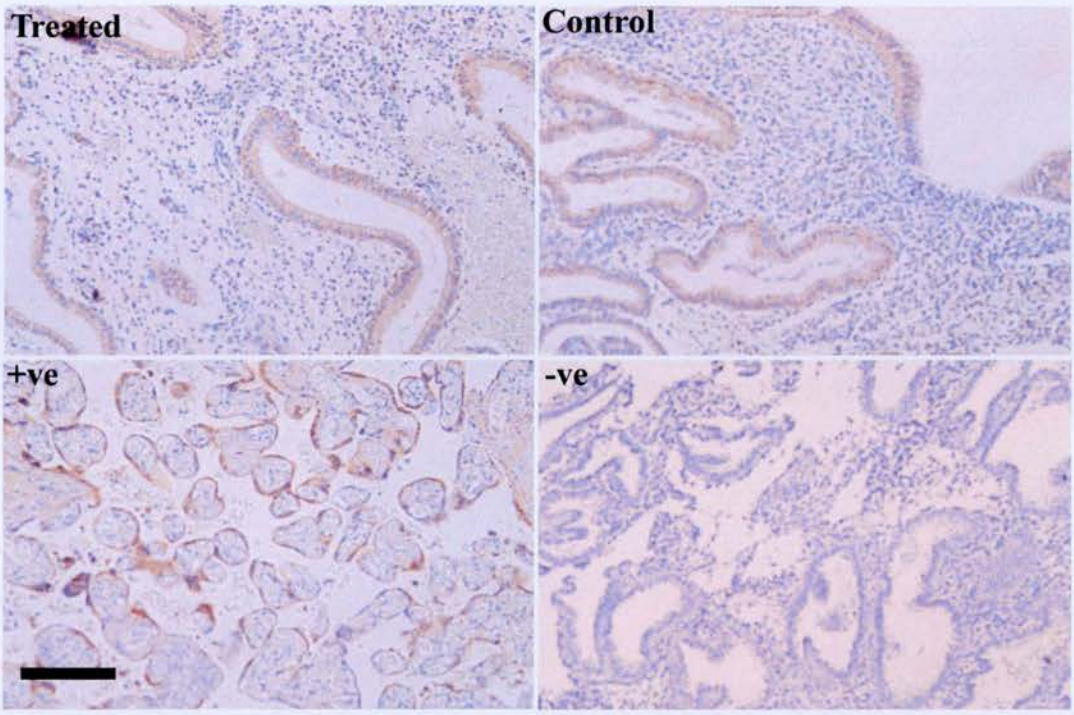


Figure 7.3 Immunolocalisation of 3βHSD protein in GnRH-exposed endometrium (treated) compared to normal mid-secretory stage endometrium (control), +ve= positive control, human placenta, -ve=negative control, pre-immune serum. Scale bar=10 microns.

7.3.3 17βHSD mRNA expression

17βHSD-2 expression was downregulated in GnRH-antagonist-treated endometrium compared to mid-secretory stage control endometrium. This was found to be statistically significant although levels were low in both treated and untreated endometrium ($p=0.02$). 17βHSD-5(AKR1C3) mRNA expression was downregulated in GnRH-antagonist-treated endometrium compared to mid-secretory stage control endometrium. There was only sufficient RNA of suitable quality left available for 17βHSD-2 transcript analysis in two samples in the study group, therefore it was not possible to perform valid statistical analysis on 17βHSD-5 data. These data are shown in Figure 7.4.

7.3.4 AKR1C3 (17βHSD-5) protein expression

AKR1C3 (17βHSD-5) protein was expressed in the glandular epithelia and endothelial cells of both GnRH-antagonist-treated and untreated endometrium. Immunoreactivity was intense in both groups and did not differ significantly between treatment and control groups. These data are shown in Figure 7.5. Charts showing immunoscores are included in Figure A14 of Appendix 1.

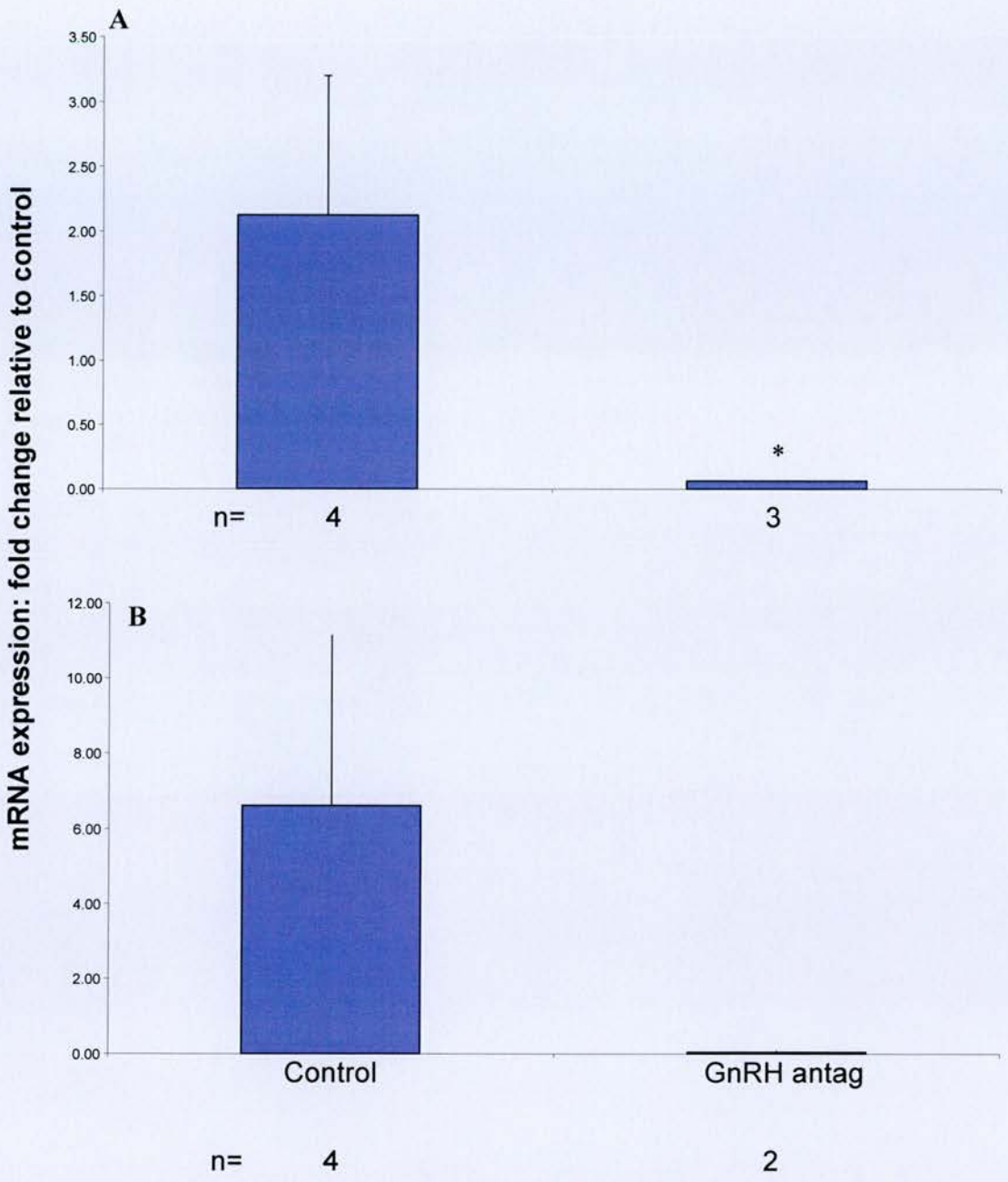


Figure 7.4 Expression of 17βHSD-2 and AKR1C3 (17βHSD-5) mRNA in GnRH-antagonist treated endometrium compared to normal mid-secretory endometrium (control), mean ± SEM. A=17βHSD-2, B=17βHSD-5.The comparator was an untreated control sample. *$p<0.05$ compared to untreated control.

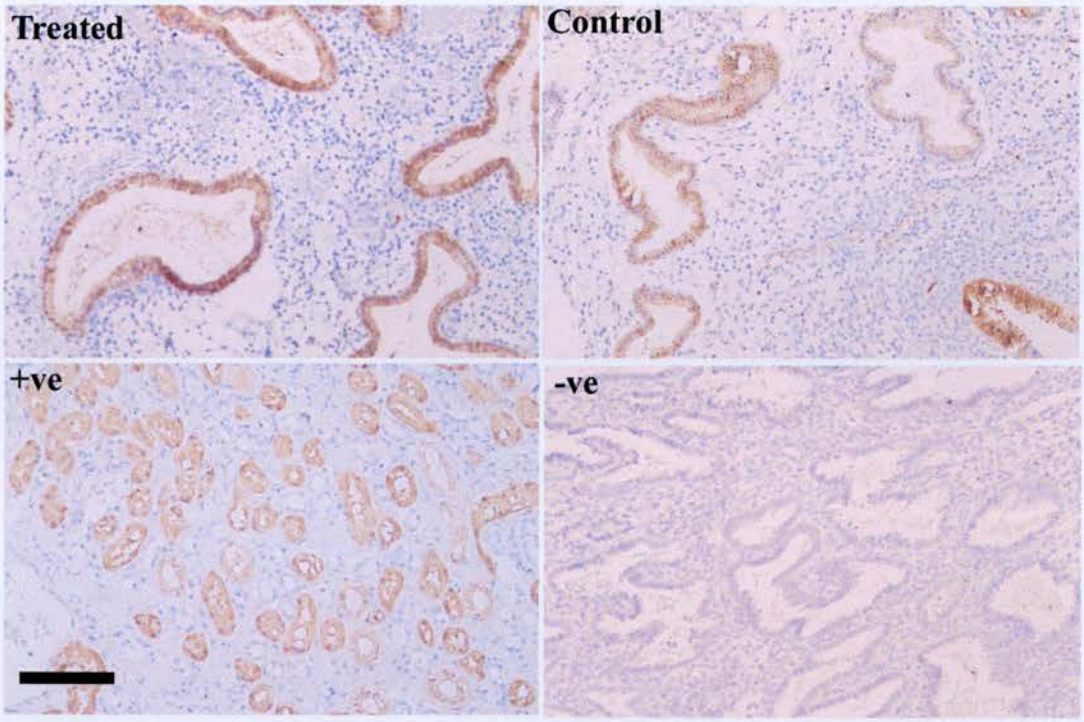


Figure 7.5 immunolocalisation of AKR1C3 (17βHSD-5) protein in GnRH-antagonist treated endometrium (treated) compared to mid-secretory endometrium (control), +ve= positive control, human kidney, -ve=negative control, primary antibody substituted for matched IgG. Scale bar=10 microns.

7.4 Discussion

FSH and GnRH antagonist treatment is a widely used regime for controlled ovarian stimulation as part of IVF/ICSI cycles. The effects of this treatment on the endometrium, especially in relation to embryo implantation, have not been investigated in detail. Histological advancement of the endometrium has been reported (Kolibianakis et al., 2002); however, there are no reports investigating sex steroid metabolism during the putative window of implantation (mid secretory phase). Thus a number of enzymes involved in regulation of sex steroid availability were investigated in endometrium that had been exposed to GnRH antagonist treatment.

3βHSD-1 and -2 are predominantly responsible for the synthesis of progesterone from the less active pregnenolone and can also produce androstenedione from less active precursors. 17βHSD-2 and 17βHSD-5 (AKR1C3) are both involved in the metabolism of androgens and estrogens. 17βHSD-2 converts active androgens and estrogens to their less potent forms, whereas 17βHSD-5 (AKR1C3) acts in the reverse direction and makes active steroids available. The relative ratio of these two enzymes may be a key factor in determining availability of sex steroids.

Both 3βHSD-1 and -2 mRNA were downregulated in GnRH antagonist treated endometrium. Levels were very low in mid-secretory control endometrium, but were lower still in GnRH antagonist treated endometrium. 3βHSD-1 was only detectable at negligible levels; however 3βHSD-2 was not detectable at all in the study group. 3βHSD

protein was localised to the glandular and surface epithelia in both control and GnRH-antagonist-treated endometrium. Expression was not found to be significantly different between the two groups. 3βHSD-1 and -2 act to synthesise progesterone from pregnenolone, thus a reduction in expression of these enzymes will lead to a downregulation in local production of progesterone. It has been proposed that a number of factors affecting implantation e.g. growth factors, hormones, cytokines are under the control of progesterone, thus this alteration may have implications for the success of IVF treatments using GnRH antagonists. However the implication of the data showing no alteration in 3βHSD protein expression between treated and untreated endometrium is that the upregulation of sex steroid hormones resulting from GnRH antagonist treatment does not have a significant effect on 3βHSD protein expression, and consequently suggests 3βHSD is not a key factor in influencing endometrial receptivity in these women. Conversely, it may be the case that immunohistochemistry is not a sensitive enough technique to detect small changes in protein expression that may be significant.

3βHSD can also act further downstream in the steroid biosynthesis pathway, converting DHEA to androstenedione, a less potent androgen. This may also have implications for the endometrium although the precise roles of androgens in the female reproductive tract are still to be determined.

The cognate receptors for these steroids have been described in normal endometrium. Lessey et al. (1988) found progesterone receptor (PR) to be detected in the glands and stroma of the endometrium, with expression greatest in the stromal cells in the secretory

phase. Studies in our laboratory (Vani et al., 2007, in press) have found PR to be downregulated in the endometrial glands and stroma following GnRH antagonist treatment. The downregulation of both the enzymes that synthesise progesterone (3βHSD-1 and -2) and PR suggest GnRH antagonist treatment causes a decrease in progesterone action in the human endometrium, which may have an effect on endometrial receptivity.

Catalano et al. (2003) showed that progesterone action on estrogen-primed endometrium e.g. following fertility treatment can make the endometrium receptive to implantation. Conversely, progesterone withdrawal returns the endometrium to a non-receptive state. The downregulation of 3βHSD and PR seen in the endometrium following GnRH antagonist treatment in these present studies would have the equivalent effect of progesterone withdrawal, thereby supporting the theory of reduced endometrial receptivity following IVF treatment with GnRH antagonists.

This study by Catalano et al. (2003) also identified 12 genes whose expression was altered following progesterone withdrawal (by RU486 treatment) in secretory endometrium. These data included genes involved in apoptosis, transcription, stress and particularly cell signalling. Some of the genes downregulated by progesterone withdrawal are members of two important signalling pathways, the JAK/STAT and JNK pathways. These pathways are used by cytokines and growth factors and represent a possible mode of action for the failure of implantation seen in GnRH antagonist IVF protocols.

Catalano et al. (2007) reported results of a further study utilising a gene microarray to identify progesterone-regulated genes. More than 500 genes were found to be altered following progesterone antagonist (RU486) administration, including chemokines, which are known inflammatory mediators (Critchley et al., 1999), MMPs, which are vital in tissue breakdown and members of the Wnt signalling pathway which has been proposed to be involved in implantation and signalling between the endometrium and embryo (Miller and Sassoon, 1998; Mohamed et al., 2005).

Expression of 17βHSD-2 mRNA was also downregulated in GnRH antagonist treated endometrium. This enzyme acts to convert estrogens and androgens into less active forms. 17βHSD-5 (AKR1C3), which carries out the reverse of the reactions catalysed by 17βHSD-2, (produces active androgens and estrogens) was also studied. Expression of both 17βHSD-2 and -5 mRNA was decreased in endometrium exposed to GnRH antagonists. 17βHSD-2 reduction was found to be significant; however it was not possible to conduct statistical analysis on the 17βHSD-5 mRNA data. There was a greater downregulation in expression at an mRNA level of 17βHSD-5 than 17βHSD-2, thus the ratio of these enzymes is likely to be altered, favouring 17βHSD-2. 17βSHD-2 acts to inactivate estrogens and androgens. Thus the predicted alteration in the balance of these enzymes would reduce the available levels of estradiol and testosterone. Estrogen is also thought to play a vital role in implantation, and the reduction in estrogen levels may lead to failure of implantation either by direct perturbation of estrogen action or by disrupting the balance of sex steroids required for endometrial receptivity. Like

3βHSD, 17βHSD-2 also acts on androgen metabolism and this role may have further effects on endometrial receptivity and implantation.

Estrogen acts via the estrogen receptor (ER), whose expression has also been well characterised in the endometrium. ERα is expressed in both glands and stroma, at lower levels in secretory endometrium than the proliferative phase (Garcia et al., 1998; Lessey et al., 1988). ERβ was described by Critchley et al. (2001a) to be expressed in the glands, stroma and endothelial cells. ERβ was also found to be downregulated in late secretory endometrium. Vani et al. (2007, in press) found ERα mRNA to be downregulated in GnRH antagonist treated endometrium, although there was no significant difference in protein expression between treatment and control groups.

The probable favouring of 17βHSD-2 action and corresponding decrease in ERα mRNA expression suggest estrogen deprivation in GnRH antagonist treated endometrium. This may contribute to the endometrial advancement reported in GnRH antagonist treated endometrium and also to the overall disturbance of the balance of sex steroid hormones and receptors required for appropriate endometrial receptivity and subsequent implantation.

There was no significant difference in 17βHSD-5 protein expression detectable between GnRH antagonist treated and untreated endometrium. This could further support the theory of the balance of 17βHSD action to be tipped in favour of 17βHSD-2 as implied by the mRNA data. The current lack of availability of a suitable antibody for

immunolocalisation of 17βHSD-2 protein has prevented confirmation of mRNA observations at a protein level.

The controls for this study were healthy fertile women. There could be an argument that this was not the best control group, as women undergoing assisted conception have altered physiological steroid levels and possibly also alterations to the endometrium. Additionally, oocyte donors received progesterone supplementation in the mid secretory phase of the cycle to mimic a true treatment cycle. This is routine in most cycles of IVF/ICSI (Beckers et al., 2002), which may add an element of bias. However the aim of this study was to try to elucidate the differences between the endometrium at the time of embryo implantation in a natural cycle and following FSH and GnRH antagonist treatment, thus this was felt to be the most appropriate control group.

There has only been one previous report on the effects of GnRH antagonist treatment on the endometrium compared to normal mid secretory endometrium (Simon et al., 2005). However this study investigated ER and PR expression following Ganirelix treatment. This is the first time local steroid ligand availability has been studied in human endometrium following GnRH antagonist treatment.

Concluding remarks

Table 7.1 Summary of enzyme expression

Enzyme	mRNA Expression	Protein Expression	Predominant Product	Activity
3βHSD-1	↓	n/a	Progesterone, testosterone	↓
3βHSD-2	↓	↔	Progesterone, testosterone	↓
17βHSD-2	↓	n/a	Androstenedione, estrone	↔
AKR1C3 (17βHSD-5)	↓	↔	Testosterone, estradiol	↔

These studies have shown a trend for downregulation in both the metabolism and action via receptors of estradiol and progesterone in the endometrium following GnRH antagonist treatment use as part of assisted conception protocols. These data may have implications for endometrial receptivity and consequently implantation rates. Further investigation is required to determine the mechanism by which local modulation of sex steroid availability and activity influence endometrial receptivity. The expression patterns of the enzymes studied in this chapter are summarised in Table 7.4, and the potential effects on sex steroid availability have been postulated.

Chapter 8:

General discussion and conclusions

8.1 Findings of this thesis

Studies in this thesis were designed to investigate steroid metabolism and pre-receptor signalling in human endometrium across the menstrual cycle. This was carried out by studying expression patterns of steroid metabolising enzymes and their cognate receptors using QRT-PCR and immunohistochemistry. Additionally, regulation of the 11βHSDs and cognate receptors was investigated *in vitro*, and the steroid metabolising enzymes were examined in two clinical situations: endometrial tissue exposed to locally delivered LNG and endometrium exposed to the consequences of administration of a GnRH antagonist.

In Chapter 3, the expression of 11βHSD-1 and -2, GR and MR in normal endometrium and first trimester decidua were investigated. These studies were conducted using QRT-PCR to measure relative levels of mRNA at each stage of the menstrual cycle, and immunohistochemistry to study protein expression and localisation in the tissues. These studies showed that 11βHSD-1 mRNA expression was greatest at the time of menstruation, and was also increased in first trimester decidua. This pattern was replicated at a protein level, although this was not found to be statistically significant. This is the first time 11βHSD-1 expression has been studied in such detail in human endometrium, and is the first report of 11βHSD-1 protein expression in this tissue. Smith et al. (1997) had previously shown there to be low levels of 11βHSD-1 activity in endometrial tissue homogenates in the presence of $NADP^+$. However, in tissue

homogenates, 11βHSD-1 is known to behave as a dehydrogenase, converting cortisone to cortisol in the presence of $NADP^+$ (Lakshmi & Monder, 1988; Bujalska et al., 2005). Thus, this study may not represent a true picture of 11βHSD-1 activity *in vivo* across the menstrual cycle. Due to the opportunistic nature of tissue sample collection, it was not possible to perform a detailed study of 11βHSD-1 activity across the menstrual cycle using carefully staged endometrial biopsies. However the expression studies conducted here allow us to make inferences about the action of these enzymes across the menstrual cycle. The greatly increased expression of 11βHSD-1 mRNA in menstrual endometrium indicated that there was a drive for increased cortisol synthesis at the time of menstruation. It is likely that this is in order to modulate the inflammatory response associated with this process. Implantation is now also accepted to be an inflammatory event, due to the remodelling associated with decidualisation. These studies showed also a large increase in 11βHSD-1 expression, both at a mRNA and protein level, in first trimester decidua, further suggesting a role for increased cortisol in order to modulate local inflammatory events.

11βHSD-2 expression was previously studied in greater depth, by Smith et al.(1997), who investigated protein expression by immunohistochemistry, and assay of enzyme activity in the endometrium. However they only compared proliferative and secretory endometrium; whereas the present study investigated the menstrual cycle in greater depth. To the best of my knowledge there are no previous reports of 11βHSD-2 mRNA expression across the menstrual cycle. Observations showed that there appeared to be greater levels of 11βHSD-2 than 11βHSD-1 at least at the level of mRNA, i.e. 11βHSD-

2 was detectable in fewer QRT-PCR cycles. It suggests that in normal, non-pregnant, non-menstrual endometrium, 11βHSD-2 dominates, restricting cortisol availability by producing cortisone. In menstrual endometrium and first trimester decidua, however, the ratio would be switched more in favour of 11βHSD-1 and cortisol production, to modulate the inflammatory response. While the present studies on the expression of 11βHSD-2 protein in human endometrium are in agreement with the findings of Smith et al. (1997), the stages of the menstrual cycle have been investigated in greater detail.

In order for glucocorticoids to elicit a response, they must be able to bind and activate a receptor. Thus, the appropriate receptor must be available. Cortisol's anti-inflammatory action occurs via a signalling pathway initiated by binding to GR. Thus, GR expression across the menstrual cycle was of interest. GR mRNA was found to be significantly increased in menstrual endometrium compared to the other stages of the menstrual cycle. This corresponds with the increased cortisol that is likely to be available following the increased 11βHSD-1 expression seen at menstruation, meaning cortisol can act to modulate the inflammatory response. GR in human endometrium has been previously studied (Bamberger et al., 2001; Henderson et al., 2003). GR mRNA has been previously reported to be expressed across the menstrual cycle with no significant difference. Neither of these studies looked specifically at menstrual endometrium however, and that is where these present studies have found significant results. However there was not a significant increase in GR mRNA expression in first trimester decidua to correspond with the increased 11βHSD-1 expression.

This is the first known report of MR expression in human endometrium. MR mRNA is expressed across the menstrual cycle. Expression is greatest in mid-secretory endometrium. This is the time when progesterone rises, in preparation for implantation. This could further enhance the earlier hypothesis that progesterone acts as an MR antagonist to prevent fetal damage. Alternatively, progesterone could be a regulator of MR expression.

MR protein is expressed throughout the menstrual cycle in the glandular epithelium, with some expression in stromal and endothelial cells. This is unlike most other nuclear hormone receptors which are localised to stromal cells (Henderson et al., 2003; Perrot-Applanat et al., 1994; Maia et al., 2001; Slayden et al., 2001). ERα and ERβ are, however, located in both the glands and stroma. More interestingly, despite being a nuclear hormone receptor, MR is predominantly localised to the cytoplasm. A previous report (Sartorato et al., 2004) showed MR to be cytoplasmic in expression, with the ability to translocate to the nucleus when ligand is bound. This may be the case here, as some nuclear staining is seen in first trimester decidua, when 11βHSD-1 levels are high. However little is known about aldosterone in human endometrium, so it is not possible to further elaborate on this hypothesis. As MR is not activated by progesterone, it would not be expected that progesterone could induce this translocation.

Chapter 4 focussed on the regulation of the 11βHSD system and associated receptors by IL-1α, and has expanded upon the work of Rae et al. (2004a,b) in our laboratory. Rae et al. (2004a) investigated the effect of IL-1α on 11βHSD-1, -2 and GR in ovarian surface

epithelial cells, and this present study aimed to expand that knowledge of cortisol signalling in cellular structures of the female reproductive tract, and investigate whether epithelial cells in a different environment behaved differently.

The present study found that 11βHSD-1 mRNA was indeed upregulated by IL-1α in a population of epithelially-enriched endometrial cells. This was suggestive that epithelial cells behave similarly in response to IL-1α, regardless of their tissue location. This hypothesis was further supported by a trend towards increased GR expression in response to IL-1α treatment in this same cell population. Although this result was not statistically significant, it was consistent with the findings of Rae et al. (2004a), who found a small but significant increase in GR mRNA expression in response to IL-1α.

A similar response to IL-1α was observed in 11βHSD-1 expression in endometrial stromal cells. Expression was upregulated in response to IL-1α treatment. However, the level of upregulation in stromal cells was roughly 5 times less than that observed in epithelially enriched cells. This is perhaps suggestive that 11βHSD-1 responds to IL-1α similarly in the different cells of the female reproductive tract, but the vast difference in the fold change in expression implies that there are also differences between the cell types. The epithelially enriched cells showed the greatest response to IL-1α, thus these are the cells which are most important in generating the 11βHSD-1 increase which leads to increased cortisol at times of inflammation.

A third cell population was studied, consisting of mixed cells sampled from near the endometrial surface with a brush. These were mainly fibroblast-like cells, with some epithelial cells. This cell population responded differently to IL-1α, in that it alone had no significant effect on 11βHSD-1 expression, however when cortisol was added, expression significantly increased. This was suggestive of a level of spatial as well as cellular control of 11βHSD-1 expression. IL-1α is a pro-inflammatory cytokine, however it has been shown to act to increase expression of the anti-inflammatory steroid cortisol. This shows the precise level of control at work in the human endometrium.

There were drawbacks with this study, in that the cell separation method was perhaps not optimal, and a pure population of epithelial cells was not available. However the difference in fold change in expression between 11βHSD-1 in epithelially enriched cells and stromal cells indicates that an epithelial cell response was measurable.

These data suggest that many, if not all, cells in the female reproductive tract respond in a broadly similar manner to IL-1α; however there are differences between cell types and tissue locations in the level of this response.

In Chapter 5 the expression of a number of enzymes involved in the metabolism of sex steroids were investigated using QRT-PCR and immunohistochemistry. The 3βHSD isoforms were investigated; these are involved in the synthesis of all steroid hormones, by catalysing the conversion of pregnenolone to progesterone. 3βHSDs are also involved in the metabolism of androgens.

Two previous studies have investigated 3βHSDs in human endometrium, a notable one being by Rhee et al. (2003). This study utilised immunohistochemical methods to detect 3βHSD in human endometrium, finding stronger immunoreactivity in secretory than proliferative endometrium. Rhee et al. (2003) only compared proliferative and secretory endometrium, so the present study investigated 3βHSD expression across the menstrual cycle in greater detail using an antibody raised against human recombinant 3βHSD-2 which similarly detects 3βHSD-1. In agreement with Rhee et al. (2003), 3βHSD was predominantly expressed in the glands. However in contrast to this earlier report, the current study found no significant difference in expression across the menstrual cycle.

Rhee et al. (2003) used RT-PCR to identify which 3βHSD isoform was present, and showed it was 3βHSD-1. 3βHSD-1 and-2 mRNA expression were measured quantitatively in the studies in this thesis and neither isoform was detectable in endometrium, consistent with the very low levels of 3βHSD protein. 3βHSD-1 mRNA was detected in first trimester decidua, thus it is likely that this is indeed the isoform detected by the antibody.

The incredibly low levels of 3βHSD protein in human endometrium suggest that there is no need for local progesterone synthesis or androgen metabolism by this enzyme in normal endometrium. However, expression is upregulated in first trimester decidua, when elevated progesterone levels are maintained, suggesting a function of 3βHSD in maintaining these elevated progesterone levels.

Additionally, the AKR1C group of enzymes were studied. This group performs a large number of reactions, controlling the expression and activity of members of all three sex steroid families. Hitherto few data have been available about expression and regulation of AKR1C enzymes in the female reproductive tract. AKR1C1-1C3 were shown to be expressed in the uterus (Penning et al., 2000), but no quantitative or spatio-temporal studies were published until 2006 when Ito et al. showed expression of AKR1C3 (17βHSD-5) in proliferative and secretory endometrium. QRT-PCR in the present study showed AKR1C1-3 to be expressed in human endometrium and first trimester decidua, however AKR1C3 (17βHSD-5) was the only member of the enzyme family whose expression differed significantly at any stage of the menstrual cycle. Its expression was upregulated in the early secretory stage of the cycle, immediately following ovulation, when circulating estrogen levels begin to fall.

Only AKR1C3 (17βHSD-5) protein expression was studied due to the lack of suitable antibodies for the other enzymes. Strong expression was detected in the vasculature, specifically in the endothelial cells. Sex steroid receptors are generally not expressed in these cells, with the exception of ERβ (Critchley et al., 2001a). Thus it is likely that AKR1C3 has a role in estrogen activation in these cells. AKR1C3 (17βHSD-5) protein was also expressed in the glandular and surface epithelia across the menstrual cycle.

The existence of a 17βHSD "switch" has been hypothesised in this chapter, in light of the expression pattern of AKR1C3 (17βHSD-5) mRNA being the reciprocal of 17βHSD-2 (Burton et al., 2003), the enzyme that catalyses the opposite reactions.

However previous studies including that of Casey et al. (1994), showed a differing pattern of 17βHSD-2 mRNA expression, and the opposing expression patterns of 17βHSD-2 and AKR1C3 (17βHSD-5) were not seen at a protein level. Thus it has not been possible to prove or disprove this hypothesis.

Chapters 6 and 7 studied the enzymes featured in previous chapters in the endometrium in two clinical situations, following usage of the levonorgestrel-releasing intra uterine system (LNG-IUS), a progestogen only contraceptive; and treatment with recombinant FSH and a GnRH antagonist as part of treatment for sub-fertility.

Many previous studies have investigated the effects of the LNG-IUS, in particular in relation to the problem of breakthrough bleeding. However the causes of this common reason for discontinued use (Findlay, 1996) are not yet fully understood. To the best of my knowledge, the work of Burton et al. (2003) is the only known report of any of the steroid metabolising enzymes of interest in the endometrium following LNG-IUS use. 17βHSD-2 expression was upregulated in the first three months of LNG-IUS use, but cellular levels then returned to normal (Burton et al., 2003).

A number of steroid metabolising enzymes were found in the present study to be upregulated in response to LNG-IUS use. Some of these enzymes have opposing effects, such as 11βHSD-1 and -2, 3βHSD-1 and AKR1C1, yet all were upregulated. This shows there is an overall increase in steroid metabolising enzymes when the LNG-IUS is used, which would lead to greater steroid metabolising activity, and potentially a disruption in

the balance of local steroid availability. This could disrupt the endometrium leading to aberrant bleeding patterns. In particular, the 11βHSD system is known to affect angiogenesis (Small et al., 2005). Both 11βHSD-1 and -2 were upregulated in this study, thus the overall balance of cortisone and cortisol would likely be altered.

These changes in mRNA expression were generally not mirrored at a protein level however, either changes were observed but were not statistically significant, or no change occurred. This may be due to a delay in translation, or simply due to the fact that immunohistochemistry as not as sensitive and quantitative a technique as QRT-PCR.

The other clinical situation studied in this thesis was that of GnRH antagonist use in fertility treatment. This study aimed to investigate the effect of this treatment on sex steroid hormone metabolising enzymes in endometrium. There are been reports of reduced pregnancy rates when this type of fertility treatment is used (Al-Inany, 2002).

The current study was carried out alongside the work of Vani et al., (2007) on the expression of sex steroid receptors in endometrium as a result of this treatment regime. Both 3βHSD-1 and -2, and 17βHSD-2 and AKR1C3 (17βHSD-5) were downregulated in endometrium that had been treated with a GnRH antagonist. No significant difference was observed at a protein level. Again, these data show that exogenous steroid treatment leads to a perturbation in sex steroid metabolising enzyme expression levels, and presumably in the steroids modulated by these enzymes. This perturbation may be in

part responsible for the problems associated with these treatments such as breakthrough bleeding and reduced implantation rates.

8.2 Conclusions

Steroid signalling is a vital part of the control of the female reproductive system, and the availability and actions of these steroids in human endometrium vary across the menstrual cycle. This variation in steroid action controls many of the key events in the human endometrium, including menstruation and implantation.

In order for steroid hormones to elicit this tight control, they themselves are tightly regulated at a pre-receptor level by a number of factors. One vital group of factors are steroid metabolising enzymes, which have been the focus of this thesis.

These studies have shown that there is a notable capacity for steroid hormone metabolism and regulation within the human endometrium. These steroid metabolising enzymes are one of the mechanisms in place that allow the tight regulation of the tissue injury and repair process that occurs in every menstrual cycle. In addition, it has been observed that exogenous steroid manipulation in clinical situations causes an alteration in many of these enzymes. These findings should provide an insight into the problems and discomfort suffered by woman using these treatments. Additionally, these studies have begun to elucidate the control of the 11βHSD enzyme system by cytokines. Future studies will expand this knowledge and enhance our understanding of the mechanisms by which these enzymes act.

8.3 Future Studies

The studies presented in this thesis have shown the expression of steroid metabolising enzymes in endometrium across the menstrual cycle, in first trimester decidua, and following treatment with exogenous steroids. Suitable antibodies were not available for immunohistochemistry to study AKR1C1, 1C2 and 17βHSD-2, hence when antibodies become available this requires to be performed. Dual immunohistochemistry to co-localise steroid metabolising enzymes and cognate receptors should be conducted to establish the interactions between them. In addition, co-localisation of 11βHSD-1 and CD56, a uNK cell marker, would inform if the increased 11βHSD-1 expression in menstrual endometrium and first trimester decidua was due to an influx of uNK cells. Studies of enzyme activity by measuring conversion of radiolabelled steroids would complete these descriptive investigations.

Following on from these studies, there are a number of functional studies that would tell us more about the role of these enzymes, such as knockouts or knock-downs in either animal models or by gene silencing in cells using RNAi. Alternatively, treatment with a GR or other receptor antagonist would allow an initial investigation of steroid hormone depletion, but would not allow evaluation of the effect of withdrawal of a particular enzyme.

The studies on regulation of the 11βHSD system are the beginning of a much wider study. The effects of other cytokines, primarily TNFα, IL-1β, IL-4, -6 and -8 on this system are to be investigated, and similar studies to be conducted on the other enzymes. Also, due to the inability to obtain a unique population of epithelial cells, the results could be confirmed by repeating the experiments using an immortalised endometrial epithelial cell line. Cell lines could also be utilised to conduct similar studies on other endometrial cell types, in particular endothelial cells and uterine natural killer cells. ELISA protein expression assays could be utilised to strengthen the existing mRNA data.

The studies on endometrium following exogenous steroid treatment provide the first data suggesting perturbation of steroid metabolism. Again, enzyme activity studies would provide functional data. Comparison with other situations involving hormone manipulation could prove useful, such as the effect of hormone replacement therapy in post-menopausal women.

The GnRH antagonist study could be expanded to conduct a direct comparison between GnRH agonist and antagonist protocols, utilising normal mid-secretory endometrium as a control.

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Appendix 1: Immunoscores

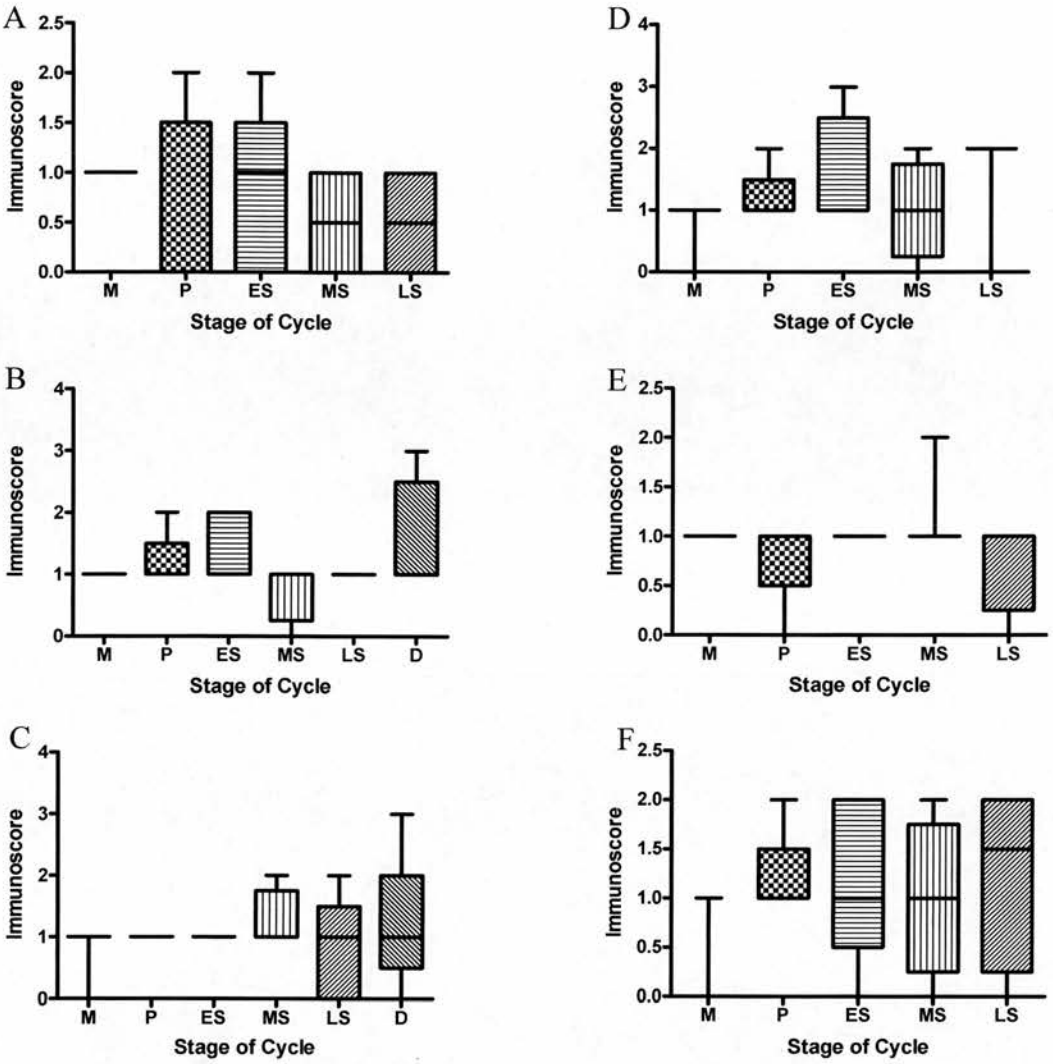

Figure A1: 11βHSD-1 protein expression across the menstrual cycle (immunoscores). Box and whisker plots: the box represents the 25th and 75th percentiles, and whiskers represent the minimum and maximum values. The heavy bar represents the median value. M=menstrual, P=proliferative, ES=early secretory, MS=mid secretory, LS=late secretory, D=first trimester decidua. A=glands in functional layer, B=stroma in functional layer, C=vessels in functional layer, D=surface epithelium, E=glands in basal layer, F = vessels in basal layer. 11βHSD-1 immunoreactivity was not seen in the stroma of the basal layer.

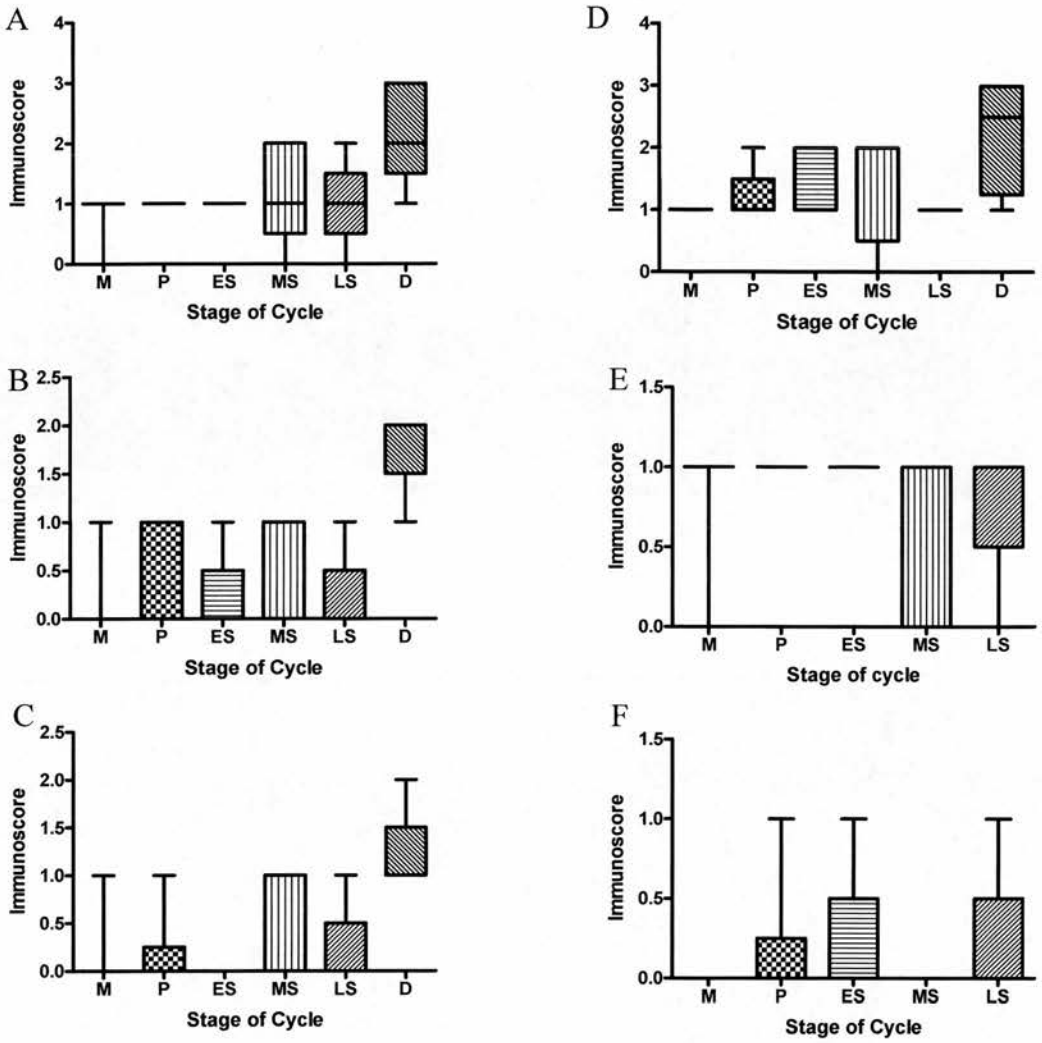

Figure A2: 11βHSD-2 protein expression across the menstrual cycle (immunoscores). Box and whisker plots: the box represents the 25th and 75th percentiles, and whiskers represent the minimum and maximum values. The heavy bar represents the median value. M=menstrual, P=proliferative, ES=early secretory, MS=mid secretory, LS=late secretory, D=first trimester decidua. A=glands in functional layer, B=stroma in functional layer, C=vessels in functional layer, D=surface epithelium, E=glands in basal layer, F= vessels in basal layer. There was no 11βHSD-2 immunoreactivity in the stroma of the basal layer.

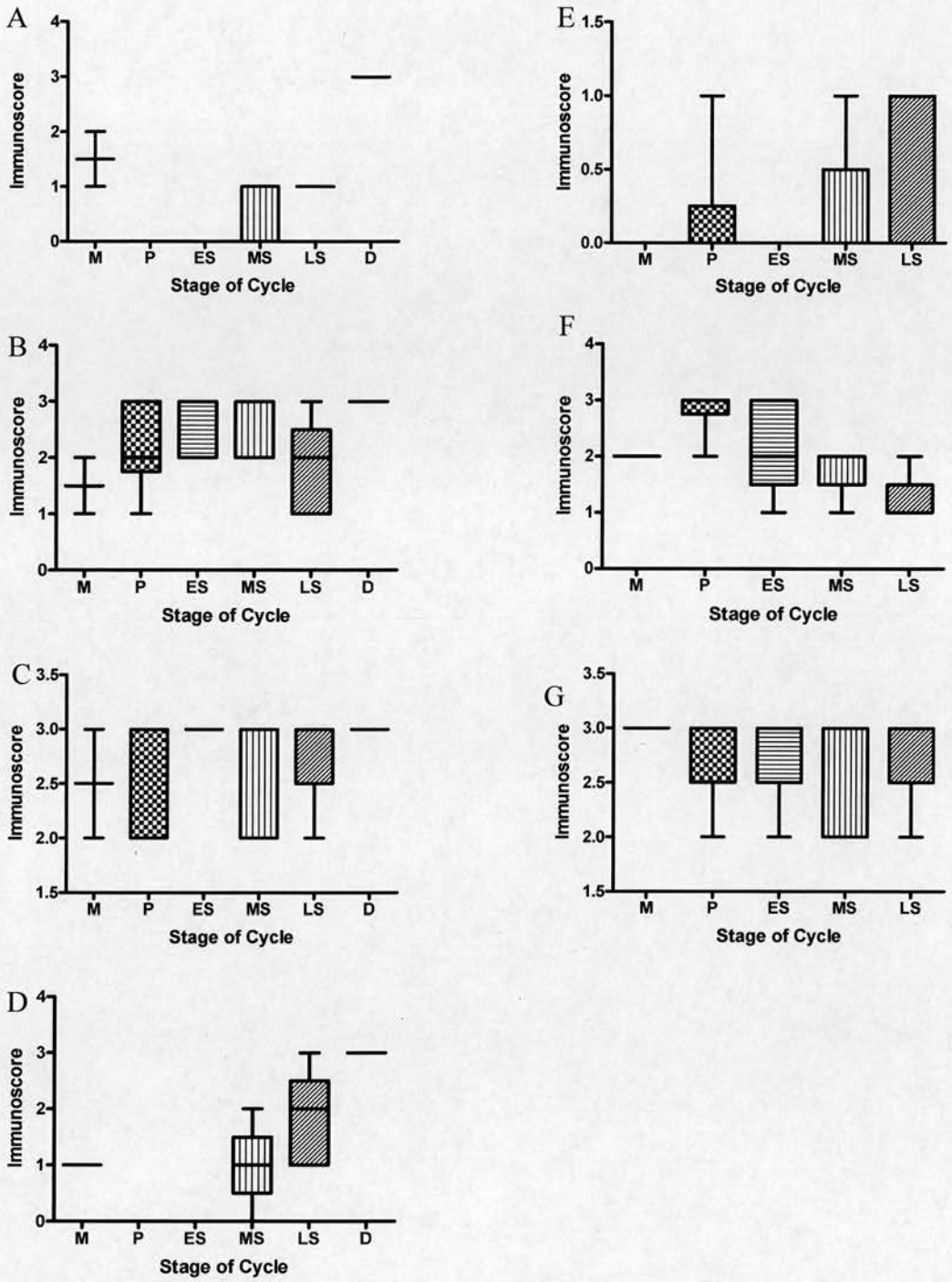

Figure A3: GR protein expression across the menstrual cycle (immunoscores). Box and whisker plots: the box represents the 25th and 75th percentiles, and whiskers represent the minimum and maximum values. The heavy bar represents the median value. M=menstrual, P=proliferative, ES=early secretory, MS=mid secretory, LS=late secretory, D=first trimester decidua. A=glands in functional layer, B=stroma in functional layer, C=vessels in functional layer, D=surface epithelium, E=glands in basal layer, F=stroma in basal layer, G= vessels in basal layer.

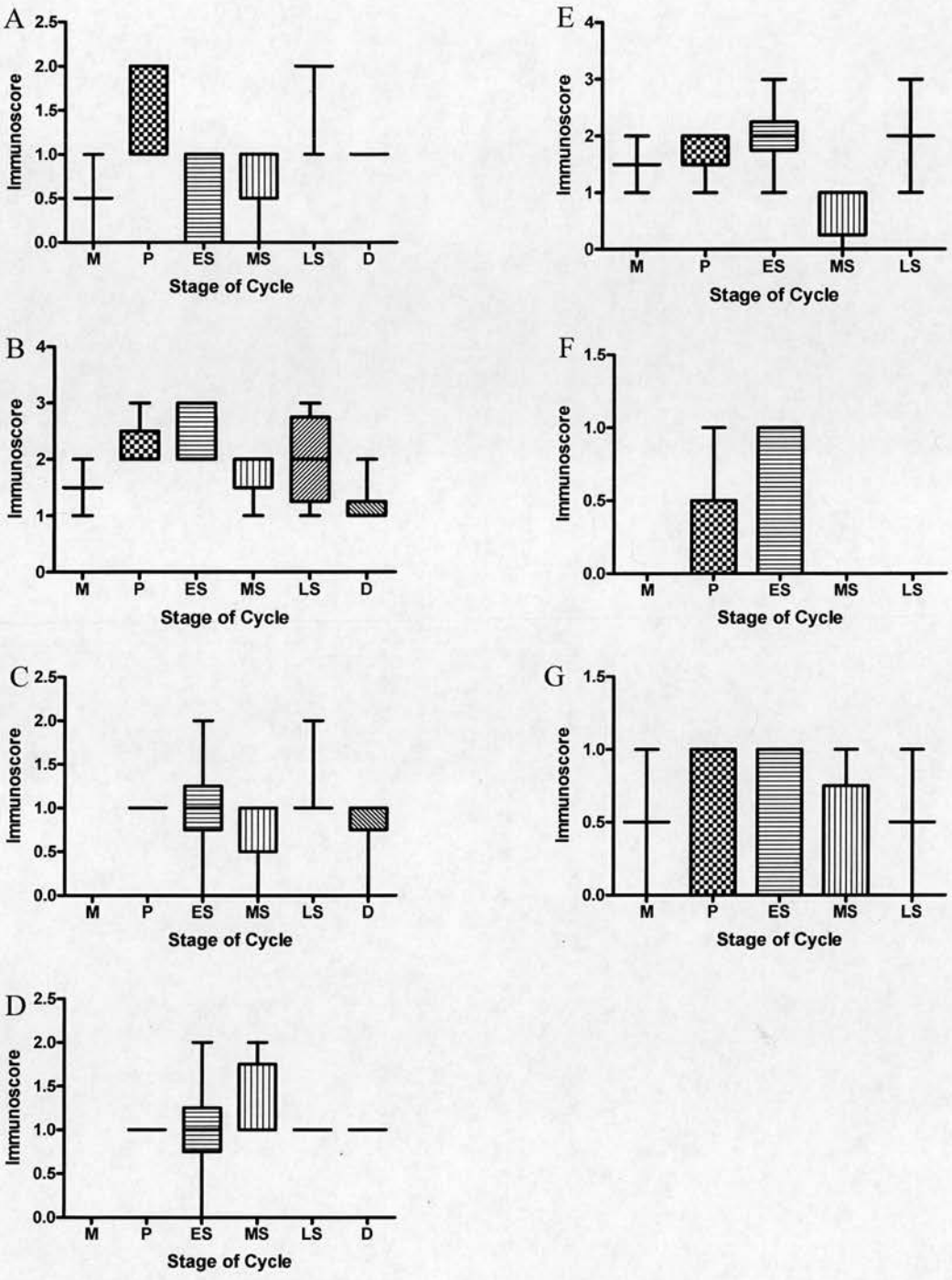

Figure A4: MR protein expression across the menstrual cycle (immunoscores). Box and whisker plots: the box represents the 25th and 75th percentiles, and whiskers represent the minimum and maximum values. The heavy bar represents the median value. M=menstrual, P=proliferative, ES=early secretory, MS=mid secretory, LS=late secretory, D=first trimester decidua. A=glands in functional layer, B=stroma in functional layer, C=vessels in functional layer, D=surface epithelium, E=glands in basal layer, F=stroma in basal layer, G= vessels in basal layer.

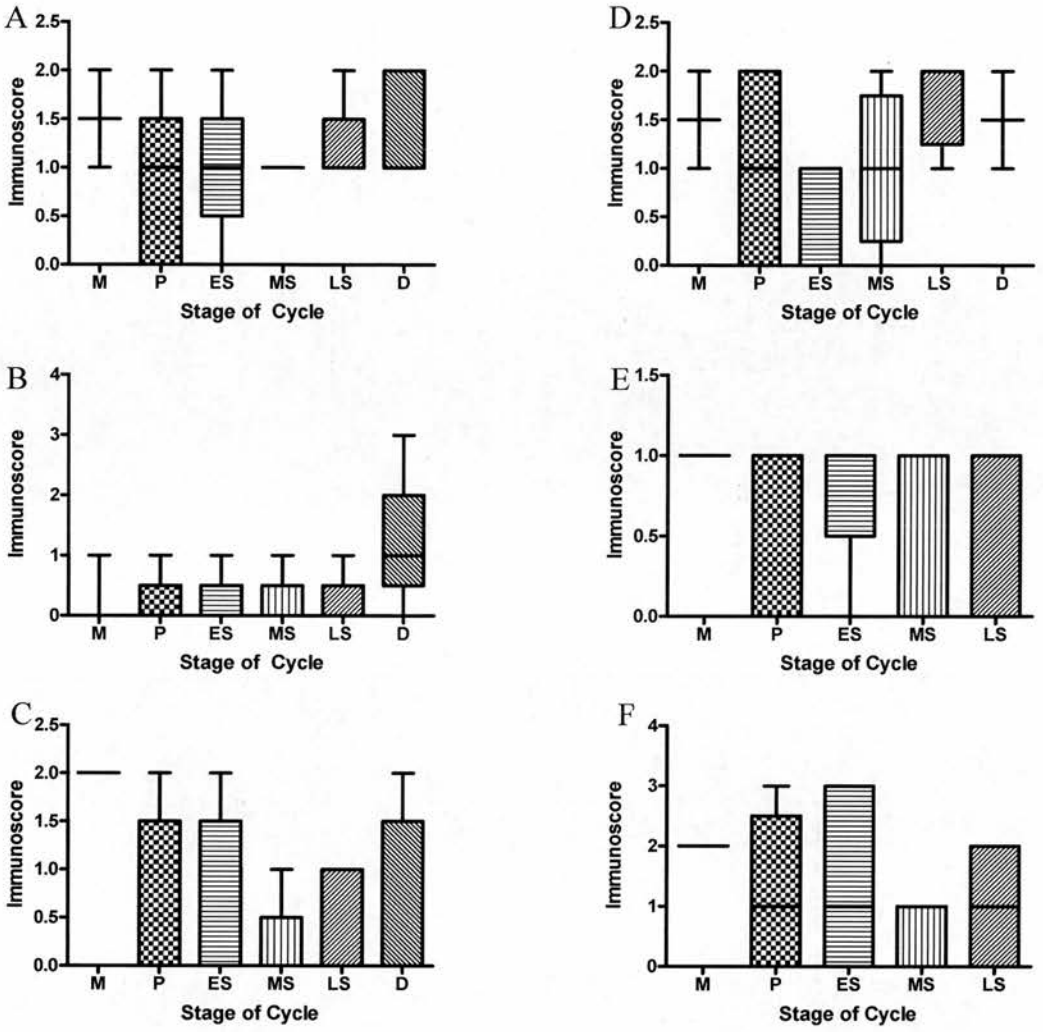

Figure A5: 3βHSD protein expression across the menstrual cycle (immunoscores). Box and whisker plots: the box represents the 25th and 75th percentiles, and whiskers represent the minimum and maximum values. The heavy bar represents the median value. M=menstrual, P=proliferative, ES=early secretory, MS=mid secretory, LS=late secretory, D=first trimester decidua. A=glands in functional layer, B=stroma in functional layer, C=vessels in functional layer, D=surface epithelium, E=glands in basal layer, F= vessels in basal layer. No 3βHSD immunoreactivity was seen in the stroma of the functional layer.

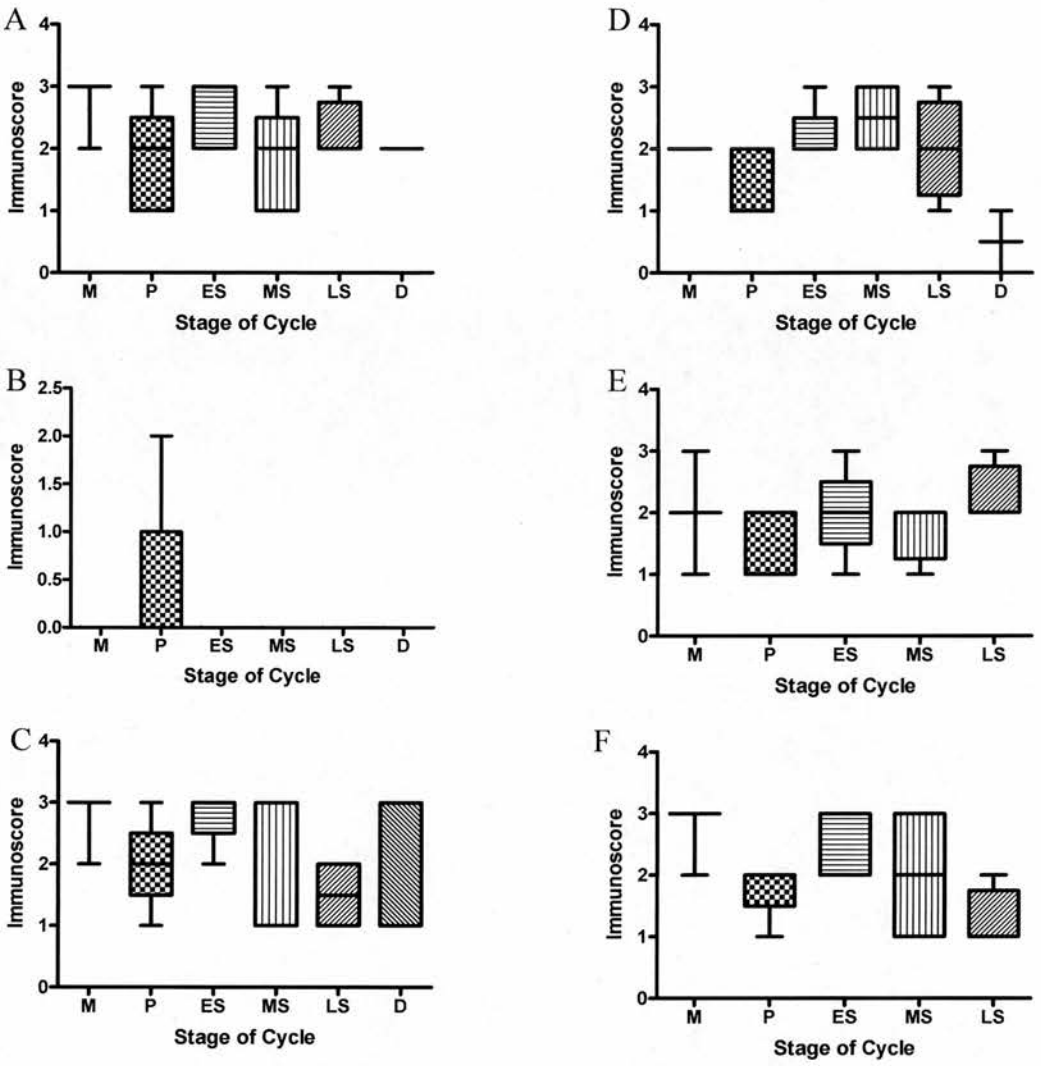

Figure A6: AKR1C3 (17βHSD-5) protein expression across the menstrual cycle (immunoscores). Box and whisker plots: the box represents the 25th and 75th percentiles, and whiskers represent the minimum and maximum values. The heavy bar represents the median value. M=menstrual, P=proliferative, ES=early secretory, MS=mid secretory, LS=late secretory, D=first trimester decidua. A=glands in functional layer, B=stroma in functional layer, C=vessels in functional layer, D=surface epithelium, E=glands in basal layer, F= vessels in basal layer. No AKR1C3 immunoreactivity was seen in the stroma of the functional layer.

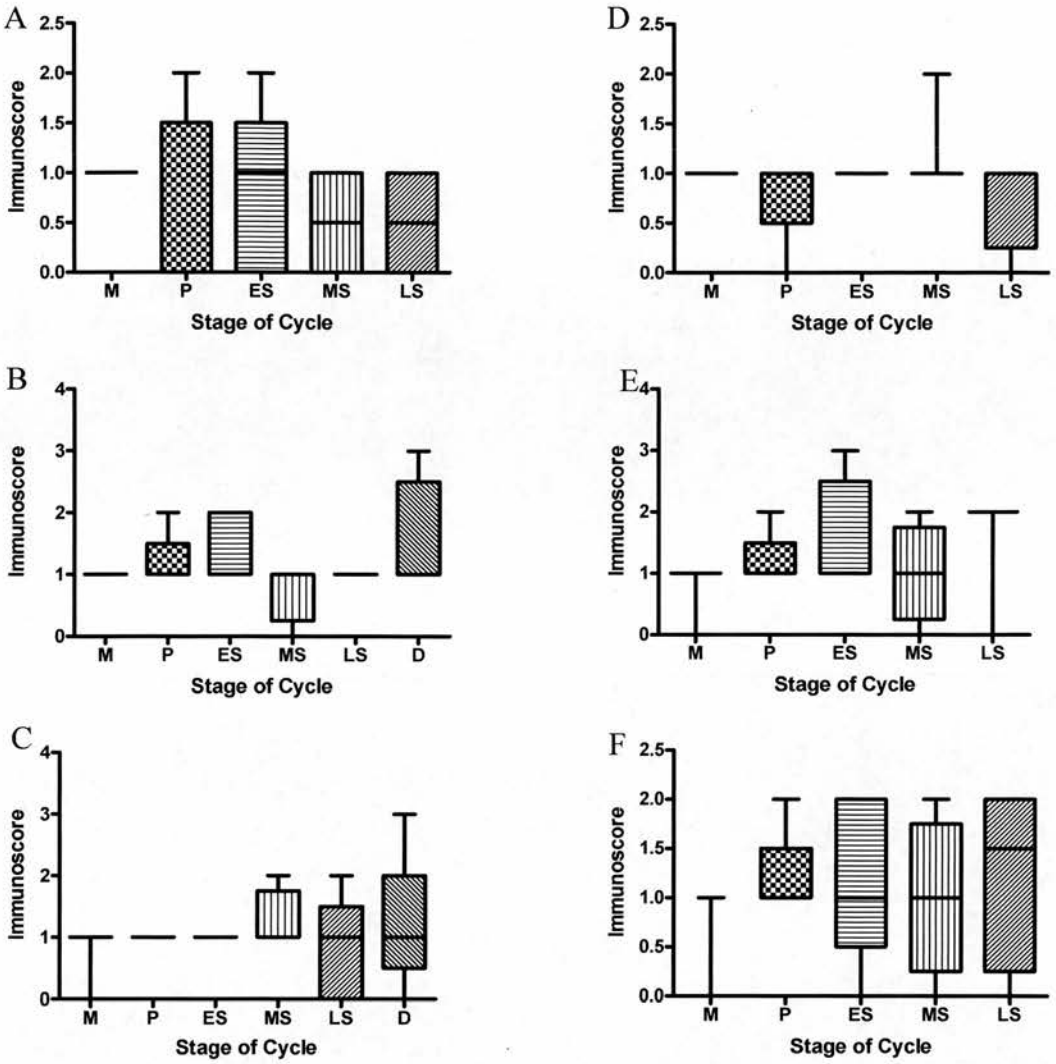

Figure A7: 11βHSD-1 protein expression across the menstrual cycle, in first trimester decidua and in pseudo-decidualised endometrium (immunoscores). Box and whisker plots: the box represents the 25th and 75th percentiles, and whiskers represent the minimum and maximum values. The heavy bar represents the median value. M=menstrual, P=proliferative, ES=early secretory, MS=mid secretory, LS=late secretory, D=first trimester decidua. A=glands in functional layer, B=stroma in functional layer, C=vessels in functional layer, D=surface epithelium, E=glands in basal layer, F=vessels in basal layer. 11βHSD-1 immunoreactivity was not observed in the stroma of the basal layer.

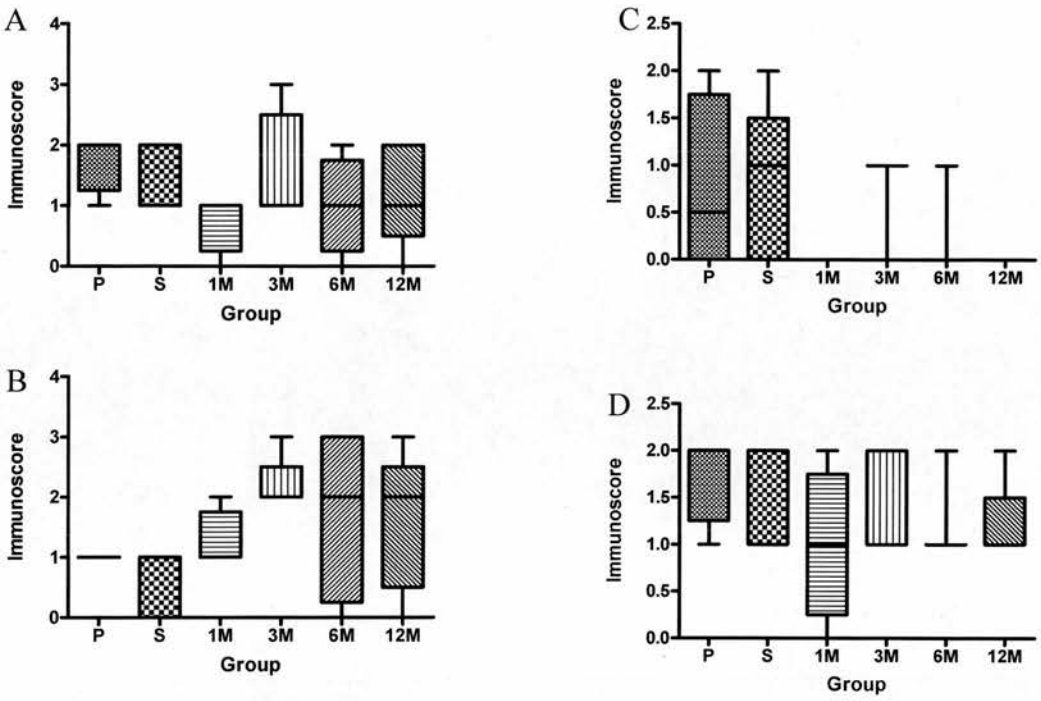

Figure A8: 11βHSD-2 protein expression across the menstrual cycle, in first trimester decidua and in pseudo-decidualised endometrium (immunoscores). Box and whisker plots: the box represents the 25th and 75th percentiles, and whiskers represent the minimum and maximum values. The heavy bar represents the median value. P=proliferative phase pre-insertion, S=secretory phase pre-insertion, 1M=1 month post-insertion, 3M= 3 months post-insertion, 6M=6 months post-insertion, 12M=12 months post-insertion. A=glands, B=stroma, C=vessels, D=surface epithelium.

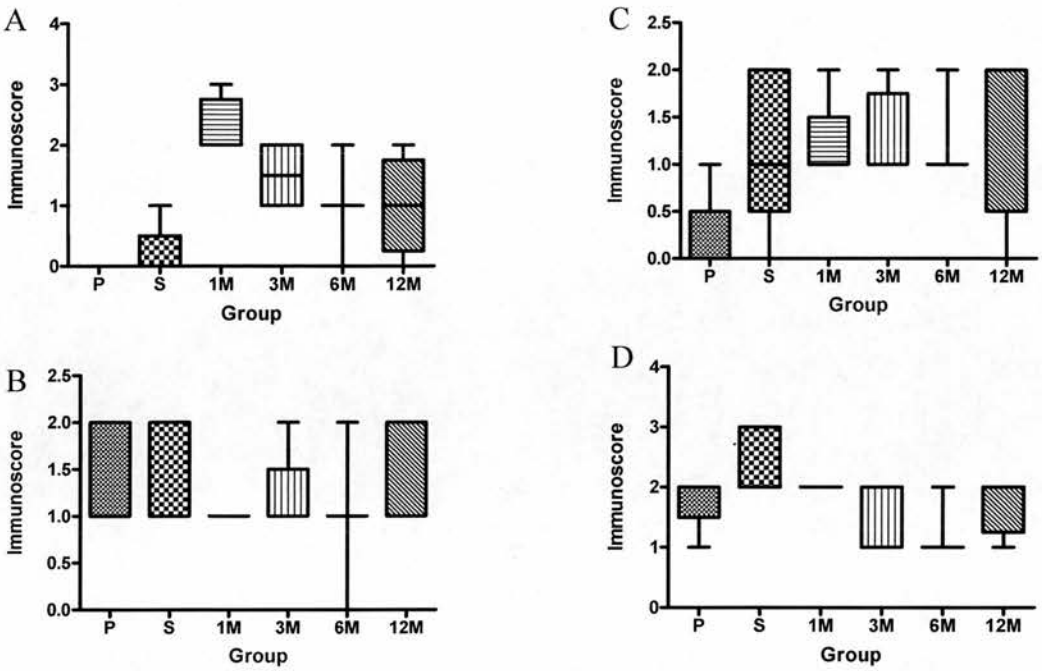

Figure A9: GR protein expression across the menstrual cycle, in first trimester decidua and in pseudo-decidualised endometrium (immunoscores). Box and whisker plots: the box represents the 25th and 75th percentiles, and whiskers represent the minimum and maximum values. The heavy bar represents the median value. P=proliferative phase pre-insertion, S=secretory phase pre-insertion, 1M=1 month post-insertion, 3M= 3 months post-insertion, 6M=6 months post-insertion, 12M=12 months post-insertion. A=glands, B=stroma, C=vessels, D=surface epithelium.

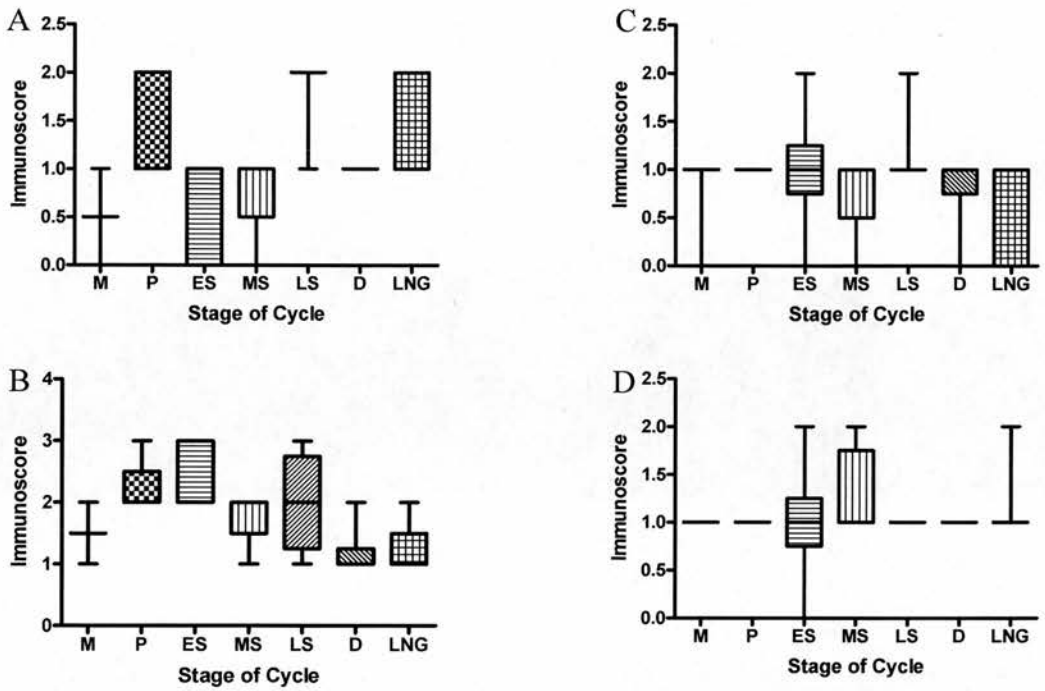

Figure A10: MR protein expression across the menstrual cycle, in first trimester decidua and in pseudo-decidualised endometrium (immunoscores). Box and whisker plots: the box represents the 25th and 75th percentiles, and whiskers represent the minimum and maximum values. The heavy bar represents the median value. M=menstrual, P=proliferative, ES=early secretory, MS=mid secretory, LS=late secretory, D=first trimester decidua. A=glands in functional layer, B=stroma in functional layer, C=vessels in functional layer, D=surface epithelium.

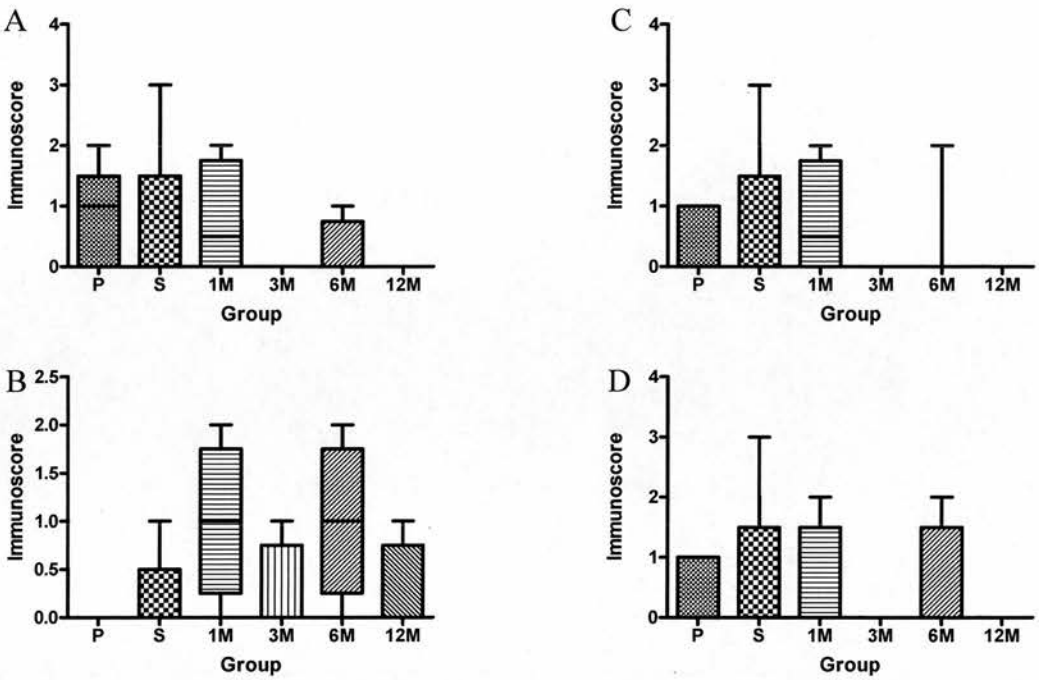

Figure A11: 3βHSD protein expression across the menstrual cycle, in first trimester decidua and in pseudo-decidualised endometrium (immunoscores). Box and whisker plots: the box represents the 25th and 75th percentiles, and whiskers represent the minimum and maximum values. The heavy bar represents the median value. P=proliferative phase pre-insertion, S=secretory phase pre-insertion, 1M=1 month post-insertion, 3M= 3 months post-insertion, 6M=6 months post-insertion, 12M=12 months post-insertion. A=glands, B=stroma, C=vessels, D=surface epithelium.

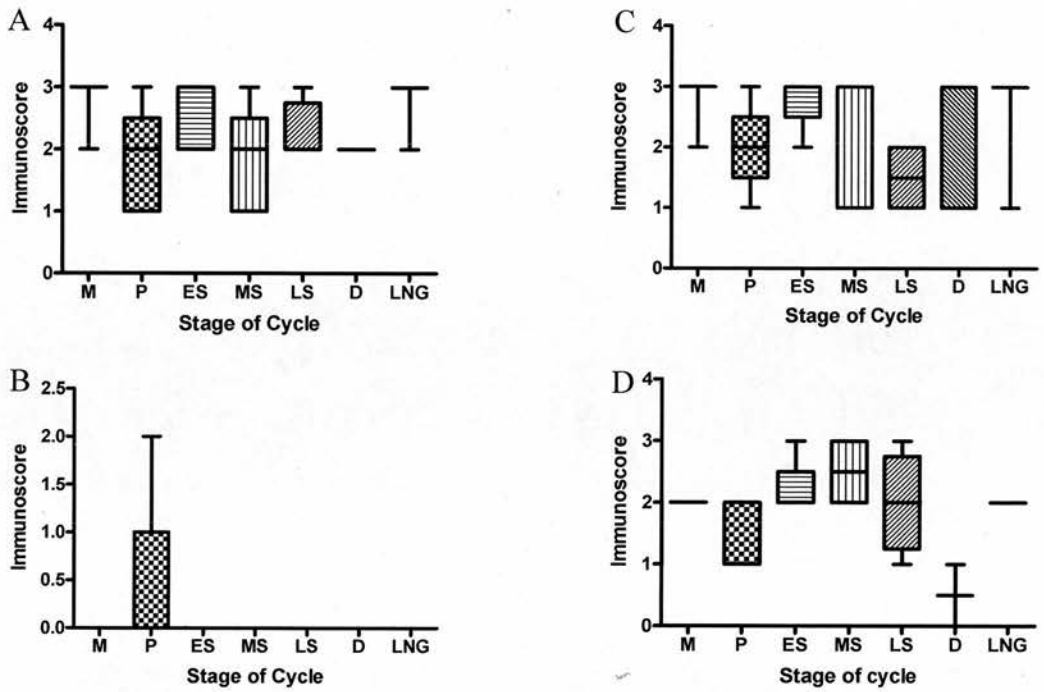

Figure A12: 17βHSD-5 protein expression across the menstrual cycle, in first trimester decidua and in pseudo-decidualised endometrium (immunoscores). Box and whisker plots: the box represents the 25th and 75th percentiles, and whiskers represent the minimum and maximum values. The heavy bar represents the median value. M=menstrual, P=proliferative, ES=early secretory, MS=mid secretory, LS=late secretory, D=first trimester decidua, LNG=pseudo-decidualised endometrium. A=glands, B=stroma, C=vessels, D=surface epithelium.

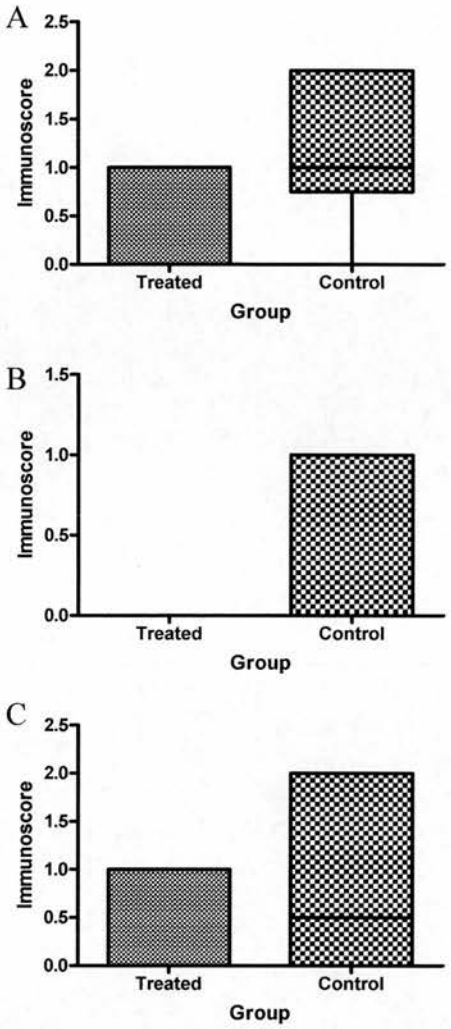

Figure A13: 3βHSD protein expression in endometrium following GnRH antagonist treatment (treated) compared to untreated mid-secretory endometrium (control) (immunoscores). A=glands, B=vessels, C=surface epithelium. No 3βHSD immunoreactivity was seen in the stroma.

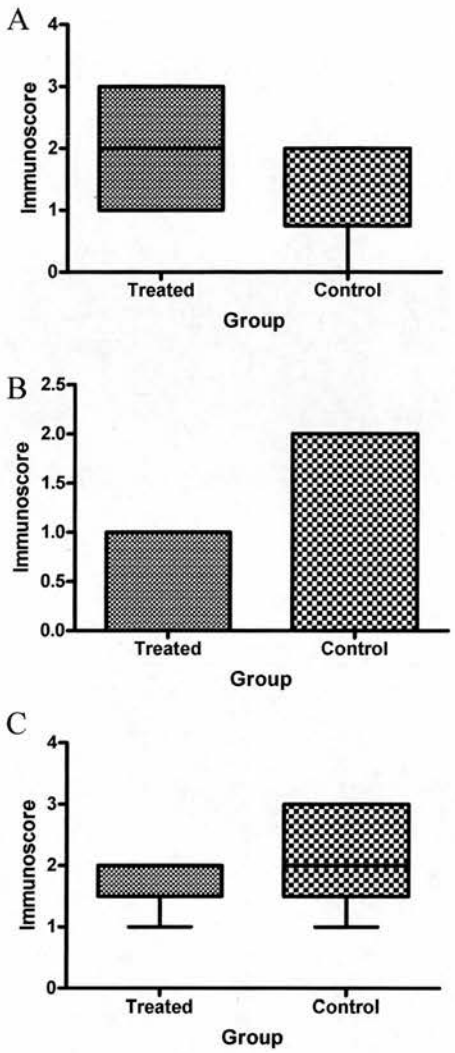

Figure A14: AKR1C3 (17βHSD-5) protein expression in endometrium following GnRH antagonist treatment (treated) compared to untreated mid-secretory endometrium (control) (immunoscores). A=glands, B=vessels, C=surface epithelium. No 3βHSD immunoreactivity was seen in the stroma.

Appendix 1:
Peer-reviewed Publications

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11β-Hydroxysteroid dehydrogenases in human endometrium

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Abstract

Key reproductive events, such as menstruation and implantation, are considered to be inflammatory processes and glucocorticoids act as anti-inflammatory agents. The balance of expression of types 1 and 2 11β-hydroxysteroid dehydrogenases (11βHSD) controls the availability of cortisol to bind to the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). Expression profiles of glucocorticoid-metabolising enzymes and their cognate receptors have been characterized in the reproductive tract. We propose that factors that peripherally promote glucocorticoid action are part of an anti-inflammatory response to tissue remodelling in human endometrium. Protein and mRNA expression in endometrium were investigated using immunohistochemistry and quantitative real-time PCR. There was up-regulated expression of 11βHSD-1 at menstruation and in first trimester decidua. 11βHSD-2 and GR were expressed across the cycle. The MR expression pattern across the cycle and in decidua implies progesterone may also play a regulatory role. The precise roles and interactions of these proteins require further investigation.

Keywords: 11β-Hydroxysteroid dehydrogenase; Endometrium; Decidua; Cortisol

1. Introduction

Ovulation, implantation and menstruation are pivotal reproductive events, all of which involve extensive tissue remodelling and subsequent repair. A number of factors mediate this remodelling, and these factors are regulated by steroids and cytokines. Steroids involved in the reproductive system include androgens, estrogens and glucocorticoids. Prereceptor signalling events and metabolism associated with these steroids are likely to be key in the regulation of such tissue remodelling.

The 11β-hydroxysteroid dehydrogenase family (11βHSDs) are enzymes that catalyze glucocorticoid metabolism, regulating the availability of active glucocorticoid (cortisol in humans and corticosterone in rats) and their inactive metabolites (cortisone and dehydrocorticosterone, respectively.) Glucocorticoids are crucial in the inflammatory response that accompanies tissue remodelling. Pro-inflammatory cytokines elicit an increased activation of cortisol from cortisone in many tissues, suggesting local cortisol activation, by 11βHSDs. This was first shown by Escher et al. (1997), in renal glomerular mesangial cells. Studies on up- and down-regulation of cortisol levels by 11βHSD-1 and -2 in inflammatory responses have been conducted at ovulation (Hillier and Tetsuka, 1998), in kidney, liver and fat (Seckl et al., 2004), and in bone (Cooper et al., 2001). Deficiencies in the regulation of 11βHSD can cause problems including liver failure, obesity, and metabolic and cardiac disorders. Thus it is likely that 11βHSDs play a similar role in the reproductive tract.

There are two characterized 11βHSDs in humans, encoded by two separate genes, located on different chromosomes, and with only 14% homology (Albiston et al., 1994). 11βHSD-1 was first identified in liver (Lakshmi and Monder, 1988) and acts predominantly as an NADPH-dependent reductase, although in the presence of $NADP^+$ it can also act as a dehydrogenase converting cortisol to cortisone. Human 11βHSD-2 was cloned in 1994 by Albiston et al. (1994) and was first identified in kidney. This enzyme is a unidirectional NAD^+-dependent dehydrogenase.

Studies have been carried out on the expression patterns of these enzymes in the female reproductive tract. The signal work on the expression of 11βHSDs in the endometrium, was conducted by Smith et al. (1997). They performed immunohistochemistry on endometrial tissue samples, collected at curettage from women with regular menstrual cycles, supported by western immunoblotting and activity assays to build an expression profile. It was found that 11βHSD-2 protein was expressed in

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0303-7207/$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2005.12.010

the luminal and glandular epithelia, with heterogeneous expression in some tissue sections. Levels of 11βHSD-2 activity were found to be higher in the secretory phase than in proliferative phase endometrium. The activity of 11βHSD-1 in endometrium was also assayed with constant levels of activity found across the cycle; however, the overall level of 11βHSD-1 activity was lower than that of 11βHSD-2.

There has been no definitive study of the expression profile of 11βHSD-1 protein in endometrium, due to the current lack of an antibody (commercial or academic) proven suitable for western immunoblotting and immunohistochemistry of endometrial samples. Arcuri et al. (1996) studied the effects of various steroid treatments on endometrial stromal cells, and found progesterone increased expression of 11βHSD-1, estrogen alone had no effect while the combination of estrogen and progesterone potentiated the effect of progesterone. They also found 11βHSD-1 up-regulation to be a feature of decidualisation in these cultured cells. Koyama and Krozowski (2001) assayed the enzymic activity of Ishikawa cells, an endometrial carcinoma cell line. They found 11βHSD activity increased in a dose-dependent manner when measured with cortisol as substrate. NAD^+ was preferred to $NADP^+$ as a cofactor, suggesting predominant 11βHSD-2 activity.

Real-time PCR (QRT-PCR) was used to study 11βHSD-1 and -2 transcripts in murine placenta and uterus (Thompson et al., 2002). 11βHSD-1 and -2 were detected in the stroma of decidua, 11βHSD-1 mRNA was expressed in the epithelial cells of the endometrium, whereas type 2 was in the stroma. 11βHSD-1 was observed in human decidua by western immunoblotting (Ricketts et al., 1998).

The expression pattern of the receptors for cortisol is also relevant. The primary receptor for cortisol is the glucocorticoid receptor (GR), but cortisol also has a high affinity for the mineralocorticoid receptor (MR). Excess cortisol binding to MR can cause disorders such as apparent mineralocorticoid excess. For this reason, 11βHSD-1 is normally found co-localised with GR, while 11βHSD-2 is commonly found in MR-expressing tissues. The tissue distribution of GR in endometrium has been reported (Bamberger et al., 2001; Henderson et al., 2003). Both studies showed GR to be expressed in the stroma and endothelial cells of blood vessels of the endometrium. Henderson et al. (2003) also found GR in the glandular epithelium of first trimester decidua. Little is currently known about the expression profile of MR in human endometrium.

2. Materials and methods

2.1. Tissue collection

Informed consent and ethical approval was obtained on all tissue samples. Full thickness endometrial tissue was collected from a number of patients undergoing hysterectomy or laparoscopic sterilisation procedures, and the stage of the cycle estimated by histological dating, reported date of last menstrual period (LMP) and serum estradiol and progesterone levels. Decidual tissue was collected from women in the first trimester of pregnancy undergoing elective termination of pregnancy. The tissue samples were fixed overnight in 10% neutral-buffered formalin at 4 °C, rinsed and stored in 70% ethanol and routinely wax-embedded. 5 μm sections were cut for immunohistochemical staining. Additional biopsies from the endometrium of the same patients were collected and stored in RNAlater (Ambion, Huntingdon, UK), an RNA stabilisation reagent used to obtain RNA.

Uterine natural killer cells were isolated as previously described. (Henderson et al., 2003). Briefly, 1×10^8 decidual cells were suspended in 300 μl buffer (PBS/2 mM EDTA/1% human AB serum). After the addition of 0.5% human γ-globulins in PBS and 100 μl CD56 magnetic cell sorting microbeads (Miltenyl Biotech, Bergisch Gladbach, Germany), the suspension was incubated at 4 °C for 20 min. The cells were washed, resuspended in buffer, and applied to a VarioMACS magnet (Miltenyl Biotech). The column was washed, and the $CD56^+$ cells were eluted and resuspended in RPMI/10% fetal calf serum. The purity of the decidual NK cells was greater than 97%, as confirmed by flow cytometry.

2.2. RNA extraction and reverse-transcriptase PCR

To obtain RNA, endometrial samples frozen at −70 °C in RNAlater were extracted using the Qiagen RNeasy Midi Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Tissue was homogenised using a hand-held homogeniser, and extraction was performed using the kit. During the extraction process the samples were treated with DNase I (Qiagen) in order to remove any contaminating genomic DNA. After extraction the RNA concentration and quality were assessed using an Agilent bioanalyzer (Agilent Technologies, South Queensferry, West Lothian, UK). The RT-PCR reaction was performed in a 10 μl volume of reaction solution containing the following: 1× Taqman RT buffer, 25 mM magnesium chloride, deoxyNTPs, random hexamers, Multiscribe reverse transcriptase, RNase inhibitor and nuclease-free water (reagents from Applied Biosystems, Cheshire, UK), and 200 ng of template RNA was added. The RT reaction was conducted at 25 °C for 60 min, 48 °C for 45 min and 95 °C for 5 min for one cycle. Samples were then stored at −20 °C.

2.3. Quantitative real-time PCR (QRT-PCR)

Oligonucleotide forward and reverse primers, and oligonucleotide Taqman probes were used to detect the sequences of interest. The probes used were commercially available, from ABI UK (Assay-on-demand), or designed as previously described (Henderson et al., 2003). Reaction mixtures were made containing ABI Taqman Master Mix, specific forward and reverse primers and probe for the gene of interest, and primers and VIC-labelled probe for ribosomal 18S RNA (Applied Biosystems). This reaction mix was then transferred to an optical plate, and the QRT-PCR reaction was performed using an ABI 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems, USA).

In the analysis of data, all values are given relative to an internal control of 18S ribosomal RNA. All samples were then compared to a proliferative endometrial sample.

Data from QRT-PCR were analysed by one-way analysis of variance.

2.4. Immunohistochemistry

Immunohistochemistry was performed by standard methods using horseradish-peroxidase-conjugated secondary antibodies, and ABC-Elite avidin biotin peroxidase complex (Vector Laboratories Inc., Peterborough, UK). Immunoreactivity was detected using the chromagen, 3,3′-diaminobenzidine (DAB). The commercially obtained primary antibodies used were against 11βHSD-2 (PC545, The Binding Site, Birmingham, UK) and GR (NCL-GCR, Novo Castra, Newcastle-upon-Tyne, UK). The MR monoclonal antibody (MRN2-2D6) was one of a series produced by immunizing mice with a peptide corresponding to aminoacids 64–82 of the rat mineralocorticoid receptor (SKEKHELLPYIQQDNSRSG-C) conjugated to keyhole limpet hemocyanin. Resulting lymphocytes were fused with SP-2-mIL6 myeloma cells. This antibody is an IgG2b isotype and cross-reacts with the human mineralocorticoid receptor.

2.5. Scoring and analysis of immunohistochemistry

Immunostaining in tissue sections was analysed semi-quantitatively on a four-point scale, where 0 = no staining, 1 = some staining, 2 = moderate staining and 3 = intense staining. All sections were scored blind by two investigators.

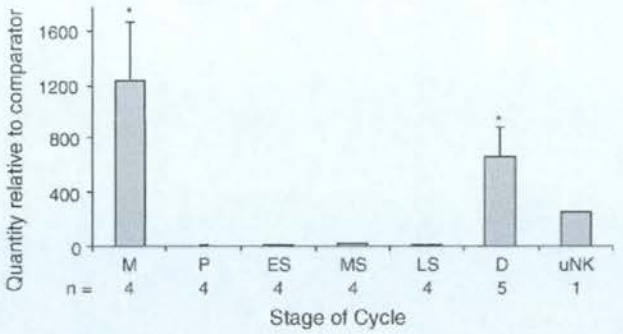

Fig. 1. Expression of 11βHSD-1 mRNA across the cycle, in first-trimester decidua, and uNK cells, mean ± S.E. M, menstrual; P, proliferative; ES, early secretory; MS, mid-secretory; LS, late-secretory; D, deciduas; uNK, uterine natural killer cells. $^{*}P<0.05$.

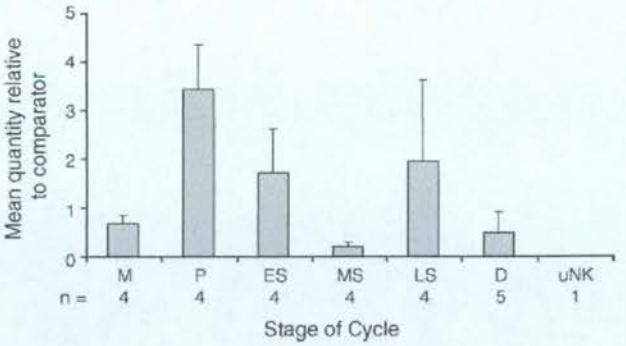

Fig. 2. Expression of 11βHSD-2 mRNA across the cycle, in first-trimester decidua, and uNK cells, mean ± S.E. M, menstrual; P, proliferative; ES, early secretory; MS, mid-secretory; LS, late-secretory; D, deciduas; uNK, uterine natural killer cells. $P>0.05$.

These results were analysed by a non-parametric method, the Kruskal–Wallis test, followed Dunn's post hoc multiple comparison test.

2.6. Western immunoblotting

Tissue was weighed, chopped roughly, placed in four volumes of homogenising buffer (10 mM Tris, 0.3 M sucrose and 1 mM EDTA, pH 7.4), then homogenised using an electric homogeniser. Tissue homogenate was aliquoted and frozen at −80 °C for later use. Protein concentration of the tissue homogenate was determined (Bradford, 1976) using a Cobas Fara centrifugal analyser and BSA as standard. The homogenate sample was diluted 2:1 with sample loading buffer containing SDS, β-mercaptoethanol, bromophenol blue and glycerol. The protein was resolved on a 10% SDS-PAGE gel and transferred to an Immobilon-P membrane (Millipore, Watford, UK). The blot was probed with the mouse monoclonal anti-MR primary antibody, followed by blocking overnight in a 3% milk solution, then horseradish peroxidase-conjugated rabbit anti-mouse IgG was applied as the secondary antibody, followed by chemiluminescence detection (Perbio Science UK Ltd., Cramlington, Northumberland, UK).

3. Results

3.1. Expression of 11βHSD-1 in human endometrium

Levels of 11βHSD-1 mRNA were measured and validated using specific Taqman primers and probes. There were very low levels of 11βHSD-1 mRNA in normal endometrium, and this did not differ significantly between proliferative and secretory phases. The levels of 11βHSD-1 mRNA in menstrual endometrium were significantly increased in comparison to in the rest of the cycle ($P<0.05$). Levels of mRNA were also increased in first trimester decidua. There was also some increase in 11βHSD-1 mRNA levels in uterine natural killer cells (uNK cells), a group of phenotypically unique cells that increase in number prior to pregnancy, in comparison to non-pregnant endometrium. These data are shown in Fig. 1.

3.2. Expression of 11βHSD-2 in human endometrium

11βHSD-2 was expressed in normal endometrium throughout the menstrual cycle at higher levels than 11βHSD-1. There was no increase in 11βHSD-2 mRNA levels in menstrual phase endometrium, unlike that seen with 11βHSD-1. Levels of 11βHSD-2 mRNA in first trimester decidua did not differ significantly from those in normal endometrium. 11βHSD-2 mRNA was not present in uNK cells. These data are shown in Fig. 2.

11βHSD-2 protein expression and localisation was studied across the menstrual cycle and in first trimester decidua. 11βHSD-2 was expressed in the cytoplasm. 11βHSD-2 protein was localised in the glandular epithelium in both the functional and basal layer of the endometrium across the cycle, and at a higher level in first trimester decidua. Low levels of the protein were seen in the stroma of the functional layer, with an increase in first trimester decidua compared to normal endometrium. 11βHSD-2 protein was not present in the stroma of the basal layer. There was negligible expression in the endothelial cells of blood vessels of the both layers across the cycle, but some expression was seen in these cells in the functional layer of first trimester decidua. There was moderate expression of 11βHSD-2 protein in the surface epithelium of normal endometrium, and higher levels in first trimester decidua. Expression did not vary in any part of the endometrium across the normal menstrual cycle. Fig. 3 illustrates 11βHSD-2 immunoreactivity studies.

3.3. Expression of the glucocorticoid receptor (GR) in human endometrium

Expression of GR mRNA was also assessed by Taqman QRT-PCR (Fig. 4). It was found that the mRNA was present in normal endometrium at low levels across the cycle, and in first trimester decidua, at levels that did not differ significantly. However, expression levels were higher in menstrual endometrium and uNK cells. This observation supports our previous findings (Henderson et al., 2003). Fig. 5 illustrates this expression pattern of GR immunoreactivity in endometrium and decidua.

GR protein was expressed in the stroma at fairly high levels. There was no significant difference in expression across the cycle or in first trimester decidua. Expression of GR was low in glandular epithelium across the cycle. There was a significant increase in expression in decidual glands. ($P<0.001$) There was high expression of GR in the endothelial in both functional and basal layers of the endometrium, and in first trimester decidua,

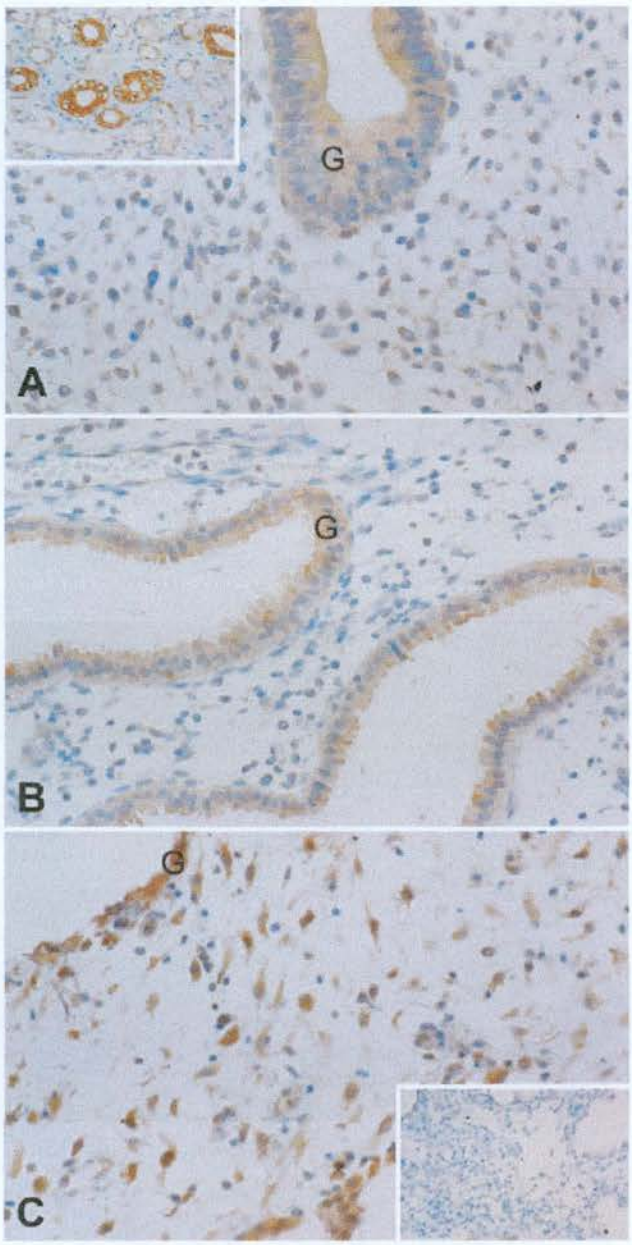

Fig. 3. Immunohistochemical staining showing expression of 11βHSD-2 in the functional layer of human endometrium and first trimester decidua brown staining represents positive 11βHSD-2 immunoreactivity, blue stain is a negative counterstain. (A) Proliferative phase endometrium, top left inset shows human kidney as a positive control, (B) Secretory phase endometrium and (C) first trimester decidua, bottom right inset shows human endometrium as a negative control. G, glandular epithelium.

with no significant difference across the cycle. Expression of GR protein increased across the cycle in the surface epithelium, from negligible expression in the proliferative and early secretory phases to weak expression in the late secretory phase. There was high expression of the protein in first trimester decidua. Fig. 5 illustrates patterns of immunoreactivity in the functional layer of the endometrium. These data again support our previous data (Henderson et al., 2003).

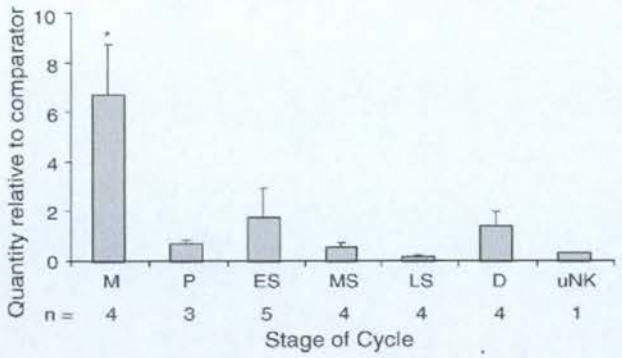

Fig. 4. Expression of GR mRNA across the menstrual cycle, in first trimester decidua and uNK cells, mean ± S.E. M, menstrual; P, proliferative; ES, early secretory; MS, mid-secretory; LS, late-secretory; D, decidua, uNK, uterine natural killer cells. $^{*}P < 0.05$.

3.4. Expression of the mineralocorticoid receptor in human endometrium

MR mRNA was expressed throughout the menstrual cycle in non-pregnant endometrium. It was also expressed in first-trimester decidua and in an isolated population of uNK cells. Expression levels are significantly higher in mid-secretory phase endometrium than in the proliferative phase, corresponding to an increase in circulating progesterone. MR mRNA levels then fell significantly in the late secretory phase, at the time of progesterone withdrawal. There was an increase in the level of MR mRNA expression in first trimester decidua, again corresponding to increased progesterone concentrations, but this result was not found to be statistically significant. Negligible MR mRNA was present in uNK cells. These data are shown in Fig. 6.

Preliminary immunohistochemical studies have shown that MR protein is expressed variably in both the nucleus and cytoplasm in different tissues. In human kidney there was MR expression in the cytoplasm, and punctate nuclear staining was also seen. In human endometrium there seemed to be predominantly cytoplasmic staining in the mid-secretory phase; however, nuclear staining was prominent in first trimester decidua. These observations are illustrated in Fig. 7. The fidelity of the antibody was confirmed by western immunoblotting of endometrial and placental samples as shown in panel D of Fig. 7 (MR has a molecular weight of 107 kDa).

The expression patterns of 11βHSD-1 and -2 enzymes, GR and MR throughout the menstrual cycle are summarised in the schematic diagram in Fig. 8.

4. Discussion

We have shown here that 11βHSD-2 enzyme is expressed in the cytoplasm of the glands in both the functional and basal layers of normal human endometrium, and also at low levels in the stroma of the functional layer. The level of expression remains constant across the menstrual cycle. Levels of 11βHSD-2 protein are increased in first trimester decidua, suggestive that 11βHSD-2 may well be involved in trophoblast invasion at the onset of pregnancy, by regulating ligand access to the GR and

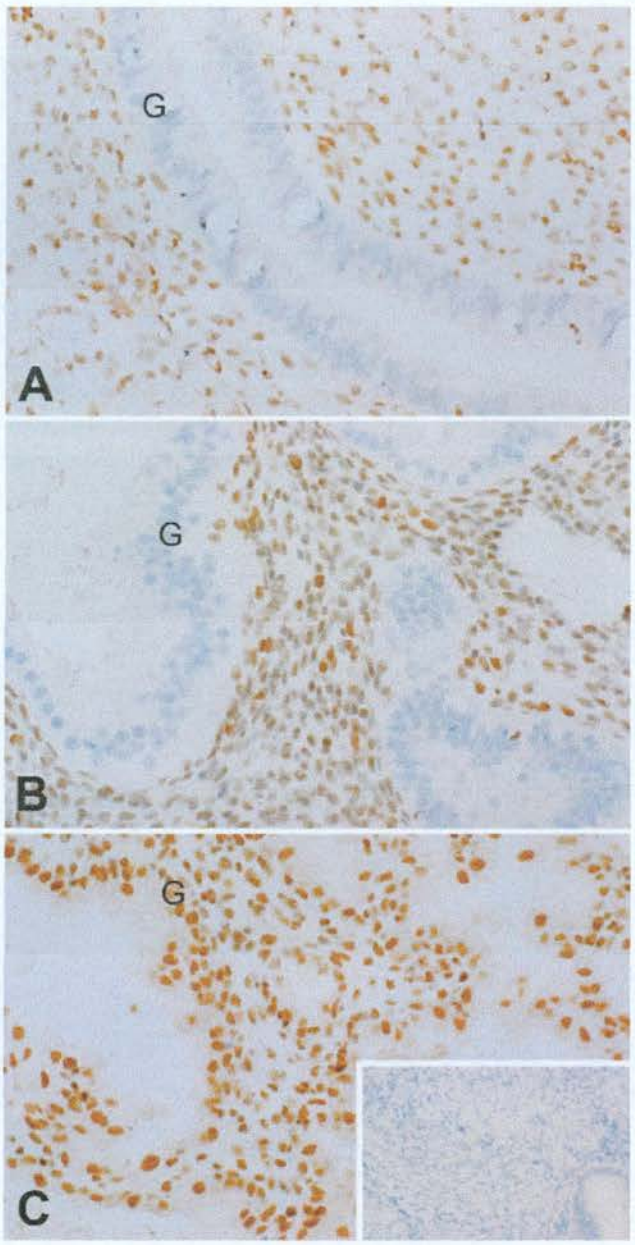

Fig. 5. Immunohistochemical staining showing expression of GR in the functional layer of human endometrium and first trimester decidua. Brown staining indicates positive GR immunoreactivity, blue stain is a negative counterstain. (A) Proliferative phase endometrium, (B) secretory phase endometrium and (C) first trimester decidua. Bottom right inset shows human endometrium as a negative control. G, glandular epithelium.

thereby preventing cortisol-mediated inhibition of matrix metalloproteinases (MMPs).

There was a large increase in 11βHSD-1 mRNA levels in endometrium at the time of menstruation in comparison to endometrium at other stages of the menstrual cycle. This may reflect an anti-inflammatory response, as both decidualisation and menstruation involve extensive remodelling of tissue. Correspondingly, levels of GR mRNA are also increased at menstruation. There may also be a link between 11βHSD-1 activity and MMP action, as proposed by Arcuri et al. (1996). It has

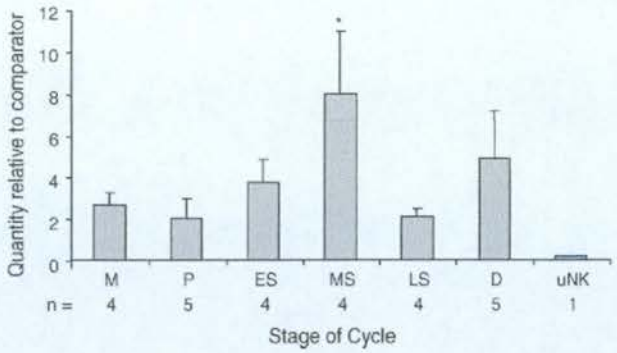

Fig. 6. Expression of MR mRNA across the menstrual cycle, in first trimester decidua and uNK cells. M, menstrual; P, proliferative; ES, early secretory; MS, mid-secretory; LS, late-secretory; D, deciduas; uNK, uterine natural killer cells. $^{*}P < 0.05$.

also been shown that MMP levels are modulated by activation of the hypothalamic–pituitary–adrenal axis, of which cortisol is a product (Yang et al., 2002). Glucocorticoids are potent regulators of extracellular matrix degrading enzymes, of which MMPs are a large group. 11βHSD-1 mRNA levels were also greatly increased in uNK cells. These are a phenotypically unique cell type that increase in number in response to progesterone in the non-pregnant secretory phase. The uNK population further increases in the first trimester of pregnancy. The increase in 11βHSD-1 mRNA expression in first trimester decidua may be due in part to expression of the mRNA in these uNK cells, a major component of the leukocyte population in first trimester decidua.

Other roles have been proposed for 11βHSDs in the endometrium and decidua. It is widely documented that 11βHSD-2 levels are high in placenta, in order to protect the fetus from high levels of circulating maternal glucocorticoids. Within the placenta, 11βHSD-2 has been localised to the syncytiotrophoblast (Krozowski et al., 1995). This is the same cell type that invades decidua in the first trimester of pregnancy, and thus may contribute to the increased levels of 11βHSD-2 seen in first trimester decidua.

11βHSDs may also be involved in the regulation of various cellular events, by modulating glucocorticoid availability, including proliferation (Bigsby and Young, 1993), apoptosis (Terada et al., 1991; Jo et al., 1993), and biosynthesis of hormones, growth factors and enzymes such as MMPs (Makrigiannakis et al., 1992; Salamonsen and Woolley, 1996). Hoffman et al. (1984) also suggested glucocorticoids may be involved in inhibition of implantation. This could explain the increase in 11βHSD-2 seen in first trimester decidua, to increase the synthesis of cortisone, and thus decrease cortisol levels, removing the inhibitory effect.

GR was expressed in the stroma across the menstrual cycle and in decidualised endometrium. Levels in the endometrial glands were up-regulated in first trimester decidua. This confirms data previously published (Henderson et al., 2003). GR is predicted to be co-localised with 11βHSD-1, thus both the ligand (cortisol) and the receptor are present in the same cells. Both GR and 11βHSD-1 mRNA levels are up-regulated in first

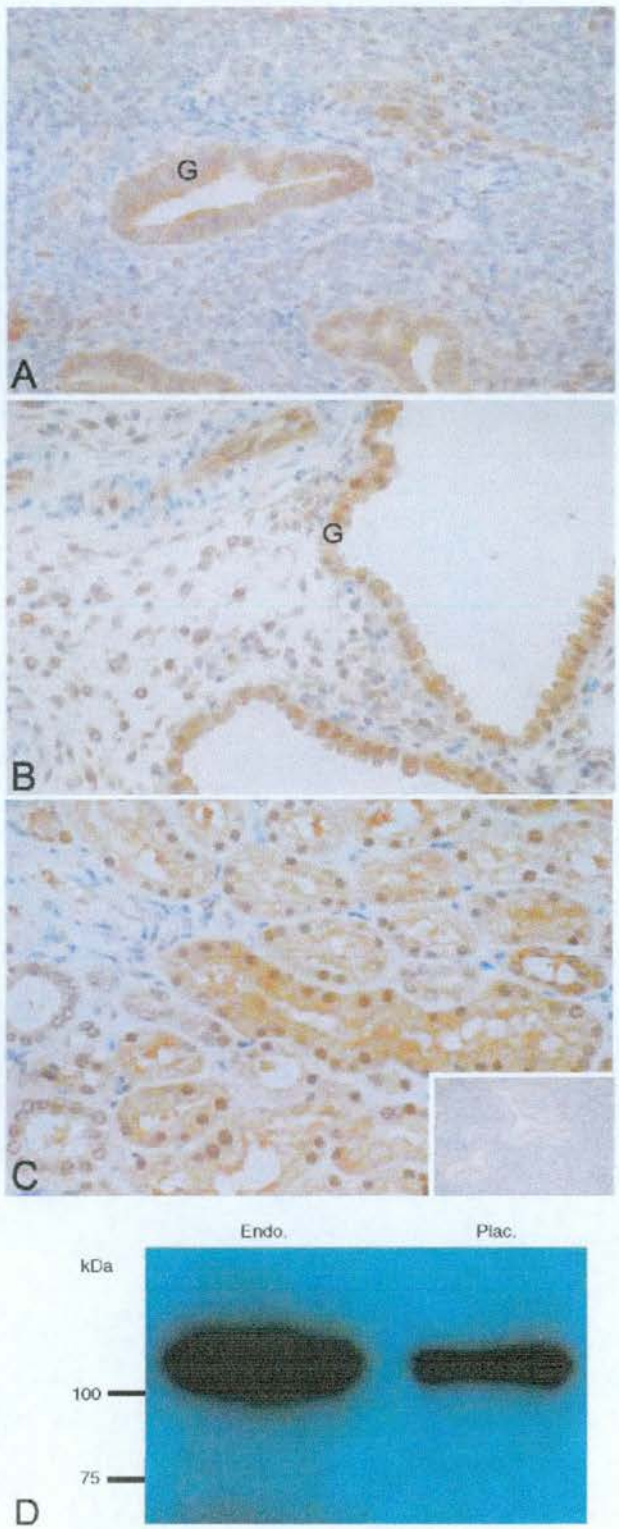

Fig. 7. Immunohistochemical staining showing expression of MR in the functional layer of human endometrium and human kidney. Brown staining illustrates positive MR immunoreactivity, blue stain is a negative counterstain. (A) Secretory phase endometrium, (B) first trimester decidua and (C) human kidney. Bottom right inset shows human endometrium as a negative control. (D) Western immunoblot showing MR (molecular weight 107 kDa) in human endometrium (50 μg protein) and placenta (20 μg protein). Endo, endometrium; Plac, placenta, G, glandular epithelium.

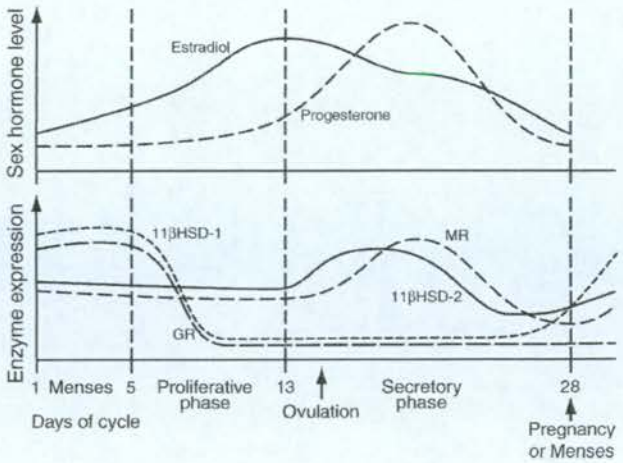

Fig. 8. Schematic diagram summarising expression patterns of 11βHSD-1 and -2 enzymes, GR and MR across the menstrual cycle.

trimester decidua, and also in menstrual endometrium. Cortisol binding to GR is a vital step in the pathway that elicits an anti-inflammatory response to tissue remodelling.

Cortisol has a greater affinity for MR than for GR, and can act as a potent mineralocorticoid when it binds to MR. Excess binding of cortisol to MR can cause a number of disorders, in particular apparent mineralocorticoid excess and hypertension. (Wilson et al., 1995, cited by Smith et al., 1997) In order to prevent this, 11βHSD-2 is normally co-localised with MR. MR mRNA studies have shown that this indeed seems to be the case. MR is expressed across the normal menstrual cycle, and in first trimester decidua, with an increase at the time of increased ovulatory progesterone concentrations, and a drop in expression when progesterone is withdrawn, suggesting MR is directly regulated by progesterone. Preliminary immunohistochemical data shows that MR expression in human endometrium is primarily cytoplasmic; however in first trimester decidua expression is both cytoplasmic and nuclear. This corresponds with the pattern of 11βHSD-1 expression, regulating the availability of ligand to bind MR. When 11βHSD-1 levels, and thus cortisol levels, are low, MR expression is predominantly cytoplasmic. In first trimester decidua, when 11βHSD-1 levels (and thus cortisol levels) are elevated, MR is seen in nuclei also. This suggests that MR translocates to the nucleus when ligand is bound, as also reported (Sartorato et al., 2004).

Interestingly, mRNA studies in uterine natural killer cells have shown that these cells express 11βHSD-1 and GR, however have negligible 11βHSD-2 or MR expression. There is currently little known about the precise role of these cells; however, it appears that glucocorticoid function is required whereas mineralocorticoid action is not. As there is very little MR present, 11βHSD-2 is not required to protect from excess cortisol binding. Previous studies have shown 11βHSD-1 mRNA and protein to be expressed in murine splenic lymphocytes (Zhang et al., 2005), and in human monocytes upon differentiation to macrophages (Thieringer et al., 2001). 11βHSD-2 was not seen to be present in either cell type.

Expression patterns of these steroid metabolising enzymes and their cognate receptors are vital in understanding pivotal

reproductive events, such as menstruation and implantation, as this information will allow development of new treatments for reproductive disorders.

Acknowledgements

This work was supported in part by MRC Programme Grant G0500047 (HODC, JIM) and a MRC Postgraduate Studentship Award (SEM). We also thank Catherine Murray and Sharon Donaldson for assistance in sample collection.

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Human Reproduction Vol.22, No.11 pp. 2981–2991, 2007
Advance Access publication on September 11, 2007
doi:10.1093/humrep/dem269

Mid-luteal endometrial intracrinology following controlled ovarian hyperstimulation involving use of a gonadotrophin releasing hormone antagonist

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BACKGROUND: There are concerns of reduced pregnancy rates with the use of gonadotrophin-releasing hormone antagonists (GnRH antagonists) in IVF/ICSI cycles. Sex steroids and their metabolizing enzymes in the endometrium may play a vital role in embryo implantation. This study has evaluated the levels and localization of sex-steroid receptors and metabolizing enzymes, 3β-hydroxysteroid dehydrogenases (3βHSD) and selected 17β-HSD (17βHSD), in mid-luteal endometrium of women treated with GnRH antagonist (Cetrorelix) and recombinant FSH (rFSH; Gonal-F) with luteal phase progesterone supplementation. METHODS: Mid-luteal phase endometrial biopsies were obtained from oocyte donors undergoing ovarian stimulation and from control women with regular periods. Immunohistochemistry and real-time quantitative–polymerase chain reaction (QRT–PCR) were used to compare protein and mRNA expression of progesterone receptor (PR), estrogen receptor α (ERα), estrogen receptor β (ERβ), androgen receptor (AR), 3βHSD1, 3βHSD2, 17βHSD2 and 17βHSD5. RESULTS: Cetrorelix–rFSH treatment caused a mid-luteal suppression of PR protein expression in the endometrial stroma, surface epithelium and glands, although expression in the glands of control samples was variable. In contrast, the treatment caused an increase in PR staining in perivascular cells. No other significant differences in protein expression were observed between the two groups. mRNA levels of AR, ERα, 3βHSD1 and 17βHSD2 were significantly reduced in the treatment group. PR mRNA levels were also reduced by GnRH antagonist–rFSH treatment, but the difference was not significant. CONCLUSIONS: Changes in the expression of sex-steroid receptors and metabolizing enzymes may lead to alterations in the activity and intracellular availability of estrogens, progestogens and androgens in endometrium of women treated with Cetrorelix and rFSH. Their impact on embryo implantation merits further evaluation.

Keywords: endometrium; GnRH antagonists; recombinant FSH; sex-steroid receptors; steroid metabolizing enzymes

Introduction

Gonadotrophin-releasing hormone antagonists (GnRH antagonists), e.g. Cetrorelix and Ganirelix, are now widely used in assisted conception treatments (Albano *et al.*, 2000; Olivennes *et al.*, 2000; The European and Middle East Orgalutran Study Group, 2001; The North American Ganirelix Study Group, 2001). They cause rapid suppression of LH levels and have been found to reliably prevent premature LH surges as a part of controlled ovarian hyperstimulation (COH) in IVF/ICSI treatment (Albano *et al.*, 2000). Their use shortens the treatment cycle and also reduces the total amount of required gonadotrophins. Furthermore, they appear to reduce the incidence of ovarian hyperstimulation syndrome (Ludwig *et al.*, 2001; Al-Inany *et al.*, 2006). However, in comparison to the 'long protocol' with GnRH agonists, with the use of GnRH antagonists there is an ongoing debate regarding pregnancy rates. Whereas some studies have found similar pregnancy rates (Ludwig *et al.*, 2001), others have raised concerns about a drop in pregnancy rates (Al-Inany *et al.*, 2006).

Despite the advances in assisted conception practices, pregnancy rates are ~20–25%. In stimulated cycles, the endometrium is exposed to supraphysiological steroid hormone levels during the follicular phase and this might be responsible for an altered steroid receptor expression profile in the early luteal phase (Papanikolaou *et al.*, 2005). After treatment with recombinant FSH (rFSH) and a GnRH antagonist, endometrial histological advancement at the time of oocyte

retrieval has been observed (Kolibianakis *et al.*, 2002). There are limited data however on the state of the endometrium during the putative window of implantation. Although not clearly defined, the window of implantation is described as from Day 5 to Day 10 after the LH surge (Sharkey and Smith, 2003).

GnRH antagonists and rFSH may impact the processes of implantation through direct effects on the endometrium or indirectly through sex steroid availability and activity.

Estrogens (Norwitz *et al.*, 2001; Ma *et al.*, 2003), progestogens (Lessey, 2003) and probably also androgens (Apparao *et al.*, 2002) are thought to play vital, but as yet not fully defined, roles in the complex mechanisms underlying endometrial development leading up to and after embryo implantation. These hormones act via their cognate receptors. An alteration of the receptor expression profile could lead to changes in the function of the respective steroid hormone.

Intracellular ligand availability could also determine endometrial receptivity. The enzyme 3β-hydroxysteroid dehydrogenase/δ5-δ4-isomerase (3βHSD) is involved in the biosynthesis of all classes of active steroids. Pregnenolone is converted to progesterone under the effect of 3βHSD in the human endometrium and this might be crucial for implantation and maintenance of pregnancy. In the secretory phase, 3βHSD is moderately expressed in the glandular epithelium of the endometrium (Rhee *et al.*, 2003).

The enzymes 17βHSD2 and 17βHSD5 have been identified in the human endometrium. The 17βHSD5 transforms not only androstenedione to testosterone and estrone to estradiol (E_2), but also progesterone to 20-hydroxyprogesterone. In the endometrium, its expression has been localized to the surface epithelium and the vascular endothelium (Pelletier *et al.*, 1999). The 17βHSD2 has a major role in the inactivation of E_2 to estrone. It is also responsible for converting androgens to less potent forms, while also activating progesterone. It is expressed in endometrial glandular epithelium, and is up-regulated by progesterone (Maentausta *et al.*, 1993). The availability of various androgenic ligands to bind to the androgen receptor (AR) may be influenced by the local presence of 17βHSD2 (Burton *et al.*, 2003). The activity of 17βHSD2 has been localized predominantly in the glandular epithelium but also in the endometrial stroma. It has been postulated that the antiestrogen action of progesterone in the endometrial glands is mediated through this enzyme (Casey *et al.*, 1994; Burton *et al.*, 2003).

The aim of this study was to compare the physiological mid-luteal endometrium with the endometrium during the putative window of implantation after treatment with rFSH and GnRH antagonists, by mimicking the exact conditions that would be expected to occur in an IVF/ICSI treatment cycle. We aimed to determine the endometrial intracrinology in relation to expression of sex-steroid receptors and steroid metabolizing enzymes during the putative window of implantation.

Materials and Methods

First, we studied the sex-steroid receptor expression during the mid-secretory phase (Table 1). Sex-steroid receptor protein expression was studied with immunohistochemistry (IHC), and the mRNA expression was studied with real-time quantitative–polymerase chain reaction (QRT–PCR). Second, we evaluated the steroid metabolizing enzymes expression during the mid-secretory phase (Table 1). Enzyme expression was studied with IHC, and mRNA expression was studied with QRT–PCR.

Patient characteristics

Institutional ethical approval was obtained and all women gave informed written consent. The study group consisted of parous women volunteers who had come forward to donate oocytes. These women underwent a cycle of ovarian stimulation as per the Edinburgh Assisted Conception Unit's protocol (Thong *et al.*, 2003). The rFSH (Gonal-F; Serono, UK) was commenced on Day 4 of the menstrual cycle. All donors commenced ovarian stimulation at the dose of 150 IU. GnRH antagonist, Cetrorelix, was commenced at a dose of 0.25 mg daily on Day 7 or 8 of their cycle once two or more follicles had reached the size of 11 mm diameter. Ovarian response was monitored by transvaginal ultrasound from Day 4. When the three largest follicles measured ≥17mm, oocyte maturation was triggered by the administration of a single s.c. injection of 10 000 U human chorionic gonadotrophin (hCG) (Profasi; Serono). Oocyte retrieval was performed 35–36 h after hCG administration. Progesterone (Cyclogest; Alpharma, UK) vaginal pessaries (200 mg) were administered 12 hourly starting 2 days after oocyte retrieval and up to the day of endometrial sampling. A pipelle endometrial biopsy (EB) was conducted 8–10 days after hCG administration.

The control group consisted of 12 healthy parous women with regular menses (25–35 days). Endometrial biopsies from eight of these women were available for the IHC studies. The women were asked to provide a urine sample on alternate days from Day 10 of their last menstrual period (LMP). Urinary LH levels were measured. An EB was performed 6–10 days after the peak of a urinary LH surge (Fig. 1). Only parous women who attended the gynaecology clinics with requests for sterilization or other complaints excluding menstrual problems or infertility were included in the control group.

From the control subjects, endometrial tissue from a subset of five parous women was available for QRT–PCR studies. In these women, the LMP, endometrial histology and serum progesterone levels were all consistent with mid-luteal phase of the cycle. This subset was chosen on the basis of mRNA quality. Since we were comparing mRNA expression in the mid-secretory phase during the putative window of implantation, it was appropriate to include mid-secretory phase endometrium from fertile women with confirmed ovulation.

Endometrial biopsies were conducted during the mid-luteal stage of the cycle (Fig. 1). Serum progesterone level was checked at the time of the EB to confirm mid-luteal stage. All endometrial samples were histologically classified as per the Noyes criteria (Noyes *et al.*, 1950). Endometrial tissue was fixed in 4% paraformaldehyde then embedded in paraffin for immunohistochemical analysis. In addition, endometrium was also frozen at the point of tissue collection in liquid nitrogen

Table 1: Details of study biopsy analyses

	Control, mean age (years) (*n*)	Study, mean age (years) (*n*)	*P*-value
Sex-steroid receptors and mRNA expression			
IHC	38 (8)	31 (5)	0.66
QRT–PCR	41 (5)	31 (4)	0.92
Steroid metabolizing enzymes and mRNA expression			
IHC	35 (6)	31 (5)	0.92
QRT–PCR	41 (4)	30 (3)	0.97

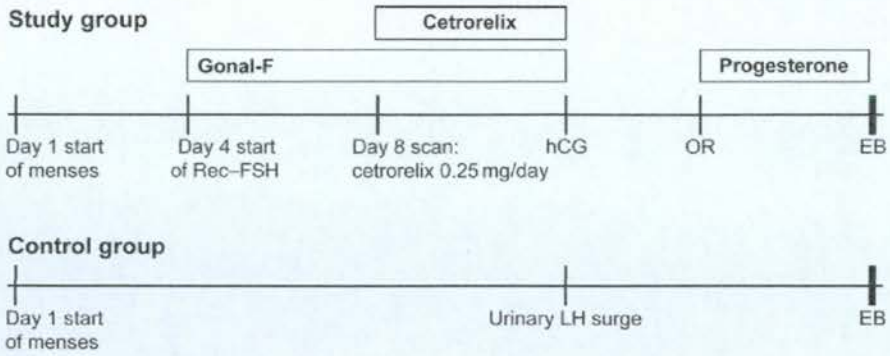

Figure 1: Timing of EB in the study and control group
OR, oocyte recovery, EB, endometrial biopsy

and stored at −70°C. RNA was extracted from frozen endometrial tissue.

The expression of progesterone receptor (PR), estrogen receptor α (ERα), ERβ and AR and enzyme expression of 3βHSD and 17βHSD5 were studied with IHC. The 3HSD antibody recognizes both human 3βHSD1 and 3βHSD2 enzymes. No documented antibodies were commercially available for 17βHSD2 enzyme and hence no immunochemistry was performed for this enzyme.

The PR, ERα, ERβ and AR mRNA and 3βHSD types 1 and 2 and 17βHSD types 2 and 5 mRNA levels were studied with QRT–PCR.

Immunohistochemistry

Paraffin sections (5 μm in thickness) were dewaxed in histoclear for 10 min and then rehydrated in descending grades of alcohol to distilled water (dH_2O). The sections were washed in dH_2O. After this, an antigen retrieval step was performed. After a 10-min wash in 0.01 M phosphate-buffered saline (pH 7.4) (PBS, Sigma, Dorset, UK), endogenous peroxidase activity was blocked. This was done by incubating the sections in 3% hydrogen peroxide in dH_2O for 10 min at room temperature. Tissue sections were then washed for 10 min in PBS. This was followed by 15 min incubation with avidin (Vector Laboratories Ltd, Peterborough, UK) at room temperature. After a rinse in PBS for 2 min, the sections were incubated with biotin (Vector Laboratories Ltd) for a further 15 min at room temperature. Following a 2-min wash in PBS, normal horse serum (NHS, Vectastatin, Vector Laboratories) was applied to each tissue section. This was followed by incubation for 20 min in a humidified chamber at room temperature. The excess serum was removed and the primary antibody was applied. The negative control for the primary antibody was substituted with mouse immunoglobulin G (mIgG1, Sigma) or rabbit pre-immune serum at the same concentration.

After the primary antibody incubation, the sections were washed between each stage for 10 min in PBS + Tween 20. The secondary antibody was then applied. To identify positive staining, the peroxidase substrate diaminobenzidine (Dako, Cambridge, UK) was used as chromogen for demonstration of epitope. Tissue sections were washed in dH_2O and counterstained with Harris's haematoxylin (a non-specific purple nuclear stain), dehydrated in ascending grades of alcohol and mounted from xylene using pertex mounting medium.

A similar protocol was used for immunostaining of all receptors. Table 2 summarizes the incubation conditions for immunolocalization of each of the epitopes studied. Commercially available antibodies were used for immunolocalization of endometrial PR, ERα, ERβ and AR. The 3βHSD rabbit polyclonal antibody was raised against recombinant human 3βHSD2 and recognizes both human 3βHSD1 and 3βHSD2 with similar affinity (S.E. McDonald and J.I. Mason,

Table 2: IHC protocols

Protein of interest	Antigen retrieval Pressure cook (PC) Microwave (MW)	Avidin–biotin pretreatment	Primary antibody	Negative control
PR	MW Buffer—0.01 M Na Citrate	No	Monoclonal mouse anti-PR antibody Novocastra, Newcastle, UK 1:40 in NHS	Mouse IgG Sigma 1:800
ERα	MW Buffer—0.01 M Na Citrate	No	Monoclonal mouse anti-ERα antibody Dako 1:400 in PBS	Mouse IgG Sigma 1:2400 in PBS
ERβ	PC Buffer—0.05 M Glycine/0.01% EDTA (pH 8)	No	Monoclonal mouse anti-ERβ antibody Serotec, Oxford, UK NRS/TBS/BSA	NRS/TBS/BSA
AR	PC Buffer—0.01 M Na Citrate	Yes	Monoclonal mouse anti-AR antibody Biogenex, CA, USA 1:240 Overnight incubation at 4°C	Mouse IgG Sigma 1:300
3βHSD	No antigen retrieval performed	Yes	Polyclonal rabbit anti-3βHSD (recognizes both isoforms) 1:500 in NGS/PBS/BSA Professor Ian Mason, University of Edinburgh	Pre-immune serum 1:500 in NGS/PBS/BSA
17βHSD5	PC Buffer—0.01 M Na Citrate (pH 6)	Yes	Monoclonal mouse anti-17βHSD-5 1:200 in NHS/PBS/BSA Professor Penning, University of Pennsylvania (Lin *et al.*, 2004)	Mouse Ig Sigma 1:300 in NHS/PBS/BSA

unpublished observations). A mouse monoclonal antibody against human 17HSD5 (Lin *et al.*, 2004) was a generous gift from Dr Trevor Penning (University of Pennsylvania, Philadelphia, USA). Although we used a mouse monoclonal against human 17βHSD2 in an earlier study (Burton *et al.*, 2003), neither this nor any commercial 17HSD2 antibody were currently available, and hence no immunochemistry was performed for this enzyme.

RNA extraction and reverse transcription

Frozen samples of endometrium stored at −70°C were homogenized and then total RNA was extracted using Trizol (Invitrogen Life Technologies Ltd, UK) according to the manufacturer's instructions. The genomic DNA was removed by subjecting the RNA to DNAse treatment. After extraction, the concentration and quality of RNA were assessed using an Agilent bioanalyzer (Agilent Technologies, South Queensferry, West Lothian, UK). The reverse transcription (RT) reaction was performed as described previously (Henderson *et al.*, 2003; McDonald *et al.*, 2006). In brief, a 10μl volume of reaction solution containing the following: 1× Taqman RT buffer, magnesium chloride, deoxyNTPs, random hexamers, Multiscribe reverse transcriptase, RNase inhibitor and nuclease-free water (reagents from Applied Biosystems, Cheshire, UK) was used. An amount of 200 ng of template RNA was added. The RT reaction was conducted at 25°C for 60 min, 48°C for 45 min and 95°C for 5 min for one cycle. An RT-negative control had template RNA but no multiscribe enzyme included, and an RT H_2O had template RNA replaced by nuclease-free water. Negative controls were included in every run. The samples were then stored at −20°C.

Quantitative real-time PCR

The primer/probe sets were designed using the Primer Express program (PE Applied Biosystems) as described previously (Henderson *et al.*, 2003) or purchased from PE Applied Biosystems' Assay on Demand service. Where possible these were chosen to span an intron to further reduce the chance of spurious readings due to genomic DNA contamination. The sequences of the primer/probe sets and their location within the specified cDNAs are given in Table 3. The 18S primers and probe were purchased from PE Applied Biosystems. A Taqman real-time PCR mix was then prepared containing final concentrations of Taqman universal PCR master mix (1x), ribosomal 18S forward and reverse primers, and probe (50 nM; PE Applied Biosystems), and forward and reverse primers (300 nM) and probe for the sequence of interest (200 nM; PE Applied Biosystems). Wells were sealed with optical caps and the PCR was run on the Perkin-Elmer ABI Prism 7900 (PE Applied Biosystems) using standard conditions.

Taqman QRT–PCR was carried out with primers and probes specific for the PR, ERα, ERβ, AR, 3βHSD types 1 and 2 and 17βHSD types 2 and 5. The validated primers and probes for 3βHSD1, 3βHSD2 and 17βHSD5 were 'Assay on Demand' products supplied by PE Applied Biosystems and these primers were intron-spanning.

Scoring, data presentation and statistical analysis of immunoreactivity

The immunostaining intensity of epitopes in all tissue sections was assessed in a semi-quantitative manner on a four point scale: 0, no staining; 1, mild/minimal immunostaining; 2, moderate immunostaining and 3, intense immunostaining. All tissue sections were scored blind by at least two observers. The semi-quantitative IHC data were analysed using the Mann–Whitney test. The QRT–PCR data were log transformed and then the *t*-test was used to test for statistical significance.

Results

Endometrial histology

In the patients treated with a rFSH–GnRH antagonist, almost all biopsies with adequate tissue showed histological features consistent with mid-secretory phase of the cycle. In one biopsy, there were features suggestive of advancement of the dates and this was reported to be consistent with early to mid-secretory phase endometrium.

We examined the immunoexpression of PR, ERα, ERβ, AR, 3βHSD and 17βHSD5 at five cellular locations, i.e. endometrial glands, stroma, surface epithelium, vascular endothelium and perivascular cells. The mRNA transcripts of PR, ERα, ERβ, AR, 3βHSD1, 3βHSD2, 17βHSD2 and 17βHSD5 were evaluated.

Table 3: Steroid receptor primer and probe sequences used for amplification by real time QRT–PCR

Primer/probe	Sequence	Position	Accession no.
AR forward	GTACCCTGGCGGCATGGT	951–1016	L29496
AR reverse	CCCATTTCGCTTTTGACACA	951–1016	L29496
AR probe	AGCAGAGTGCCCTATCCCAGTCCCA	951–1016	L29496
ERß1 forward	CCTGGCTAACCTCCTGATGCT	1459–1480	AB006590
ERß1 reverse	CCACATTTTTGCACTTCATGTTG	1529–1552 (r)	AB006590
ERß1 probe	AGATGTTCCATGCCCTTGTTACTCGCA	1499–1525 (r)	AB006590
ER forward	TGATTGGTCTCGTCTGGCG	1523–1541	NM_000125
ER reverse	CATGCCCTCTACACATTTTCCC	1602–1624 (r)	NM_000125
ER probe	TGCTCCTAACTTGCTCTTGGACAGGAACC	1572–1600	NM_000125
PR forward	CAGTGGGCGTTCCAAATGA	2151–2170	NM_000926
PR reverse	TGGTGGAATCAACTGTATGTCTTGA	2209–2233 (r)	NM_000926
PR probe	AGCCAAGCCCTAAGCCAGAGATTCACTTT	2170–2199	NM_000926
17βHSD-2 forward	TGTCAGCAGCATGGGAGGA	731–803	L11 708
17βHSD-2 reverse	GGTCACAGCCGCCTTTGAT	731–803	L11 708
17βHSD-2 probe	CCCCAATGGAAAGGCTGGCATCTT	731–803	L11 708
3βHSD1	Assay on demand		Hs00426435_m1
3βHSD2	Assay on demand		Hs00605123_m1
17βHSD5(AKR1C3)	Assay on demand		Hs00366267_m1

The positions of the sequences are given within the cDNA identified by the accession number; r denotes reverse strand.

Table 4: Immunohistochemical semi-quantitative scores

	Glands	Stroma	Surface epithelium	Vascular endothelium	Perivascular cells
PR	0.003*,[a]	0.019*,[a]	0.019*,[a]	>0.99	0.023*,[b]
ERα	0.057	0.213	>0.99	No immunoreactivity seen	0.769
ERβ	0.523	0.063	0.694	0.271	0.446
AR	0.379	0.379	0.826	0.464	0.464
3βHSD	0.235	No immunoreactivity seen	0.522	0.361	No immunoreactivity seen
17βHSD5	0.465	No immunoreactivity seen	0.411	0.404	No immunoreactivity seen

*$P < 0.05$ denotes statistical significance (Mann–Whitney test).
[a]PR was down-regulated in endometrial glands, stroma and surface epithelium of women treated with rFSH and GnRH antagonist.
[b]PR was up-regulated in the perivascular cells in the endometrium of women treated with rFSH and GnRH antagonist.

Immunohistochemistry

Sex-steroid receptors

PR immunoexpression (Table 4, Figs 2A and 3) Compared with endometrium from untreated women, endometrium exposed to Gonal-F and Cetrorelix showed significantly reduced immunoexpression in stroma ($P < 0.05$) and surface epithelium ($P < 0.05$). Conversely, PR immunoreactivity was significantly increased in the perivascular cells ($P < 0.05$). The difference in PR immunoexpression in the glands was significant ($P < 0.05$), but we observed a marked variability in expression among the biopsies in the control group. *ERα immunoexpression* (Table 4, Figs 2B and 3) Mild immunoexpression of ERα was observed at most cellular locations. No ERα immunoexpression was observed in the vascular endothelium. No significant differences were observed in endometrial ERα immunostaining between the two groups of women. *ERβ immunoexpression* (Table 4, Figs 2C and 3) ERβ immunoexpression at most cellular locations was consistently strong with or without treatment with GnRH antagonist and rFSH. Stromal expression was less intense, but overall no significant differences were observed in endometrial ERβ immunoexpression between the two groups of subjects. *AR immunoexpression* (Table 4, Fig. 2D) AR immunoexpression was negligible in the glandular epithelium with or without COH treatment. Stromal immunoexpression of AR was moderate, but overall no significant differences were observed in endometrial AR immunostaining between the two groups of subjects.

Steroid metabolizing enzymes

3βHSD immunoexpression (Table 4, Figs 2E and 4)

Irrespective of COH, expression of 3βHSD in the glands, surface epithelium and endothelium was at a low level. No immunoexpression was seen in stroma or in perivascular cells. No significant differences were observed in endometrial 3βHSD immunostaining between the two groups of subjects.

17βHSD5 immunoexpression (Table 4, Figs 2F and 4)

Moderate to intense 17βHSD5 immunoexpression was observed in the endometrial glands, and surface epithelium with or without treatment. No immunoexpression was observed in stroma, however, negligible immunoexpression was observed in perivascular cells. Moderate immunoexpression was observed in the vascular endothelium. No significant differences were observed in endometrial 17βHSD5 immunostaining between the two groups of subjects.

Quantitative real-time PCR (Table 5, Fig. 5)

Sex-steroid receptors

QRT–PCR demonstrated a statistically significant reduction in the amount of endometrial ERα mRNA ($P = 0.02$) and AR mRNA ($P=0.01$) in COH treated women compared with the controls. Although the level of PR mRNA was reduced in the COH group, the difference was not significant ($P = 0.12$). No significant differences were observed in the amount of ERβ mRNA ($P = 0.96$) between the two groups.

Steroid metabolizing enzymes

The 3βHSD1 mRNA ($P = 0.01$) and 17βHSD2 mRNA ($P = 0.02$) levels were significantly reduced in the COH group. In relation to 3βHSD2 and 17βHSD5 QRT–PCR, sufficient RNA was only available in 1 and 2 study samples, respectively. Hence in these cases, the sample size was too small for analysis. However, expression of adrenal/gonadal-specific 3βHSD2 transcripts is predicted to be minimal in endometrium and the relative 3βHSD1/3βHSD2 transcript ratio observed in the one sample is supportive of a minimal 3βHSD2 contribution to endometrial 3βHSD.

Discussion

The use of rFSH and a GnRH antagonist is an effective and reliable regime for controlled ovarian stimulation as a part of IVF/ICSI treatment cycles. However, its effects on the endometrium especially in relation to embryo implantation have not been evaluated in detail. Although histological advancement of the chronological stage of the endometrium has been observed at the time of oocyte retrieval (Kolibianakis *et al.*, 2002), the endometrial sex-steroid receptor protein and mRNA expression levels during the putative window of implantation, to our knowledge, have not been reported. Furthermore, we are unaware of any reports describing expression

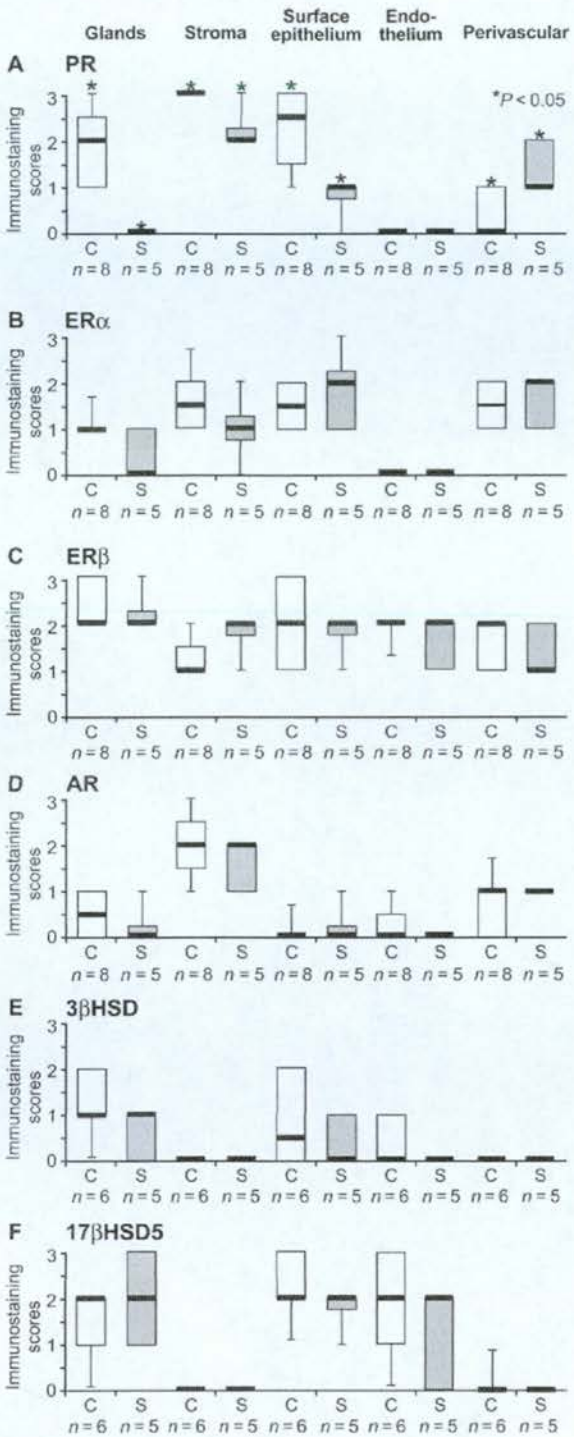

Figure 2: Immunoreactivity scores in endometrial glands, stroma, surface epithelium, endothelium and perivascular cell compartments of women in the mid luteal phase of cycle (control group) and in women who have received COH and a GnRH antagonist (study group) (**A**) PR, (**B**) ERα, (**C**) ERβ, (**D**) AR (**E**) 3βHSD enzyme and (**F**) 17βHSD5 enzyme. Note decreased PR in endometrial stroma ($P = 0.019$), surface epithelium ($P = 0.019$) and glands ($P = 0.003$), and increased PR in perivascular cells ($P = 0.023$). Box-and-whisker plots: box represents the 25th and 75th percentiles, whiskers represent the 10th and 90th percentiles and the heavy bar represents the median. C, control; S, study

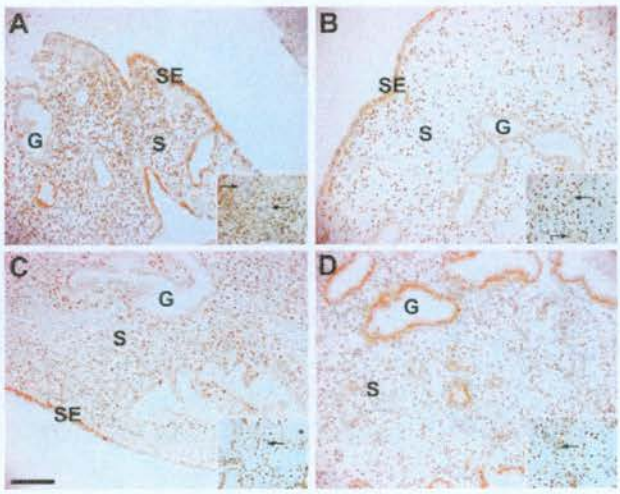

Figure 3: Immunohistochemical localization of steroid receptors in human endometrium of women in the mid-luteal phase of cycle (control group) and in women who have received rFSH and a GnRH antagonist (study group)
(**A**) PR immunostaining in endometrium from untreated women in mid-luteal phase—intense stromal (S) and surface epithelial (SE) immunoreactivity (A) inset: note low level of perivascular immunostaining (arrows). (**B**) PR immunostaining in the endometrium from women treated with a GnRH antagonist and rFSH—reduced stromal and surface epithelial immunostaining. (B) inset: more intense perivascular cell immunostaining (arrows). (**C**) ERα immunostaining in endometrium from women treated with rFSH and a GnRH antagonist—mild immunostaining at all cellular locations. (C) inset: absent immunostaining in the vascular endothelium (arrow). (**D**) ERβ immunostaining in endometrium from women treated with rFSH and a GnRH antagonist—strong immunoreactivity in all cell types. (D) inset: strong ERβ immunostaining in vascular endothelium. Scale bar = 20 microns. G, glands

of steroid metabolizing enzymes and their mRNA transcripts during the window of implantation in the mid-luteal phase of the cycle.

Here, we report a significant difference in PR protein expression in women treated with rFSH and GnRH antagonist. Significant down-regulation of PR protein expression in the endometrial stroma and surface epithelium was observed. A significant up-regulation was observed in the perivascular cells in women treated with Cetrorelix and Gonal-F. PR immunoexpression in the endometrial glands was reduced in the treatment group, although the expression in the controls was variable. No significant differences were observed in the protein expression of ERα, ERβ, AR, 3βHSD or 17βHSD5. Quantitatively, there was a significant reduction in the levels of ERα mRNA, AR mRNA, 3βHSD1 mRNA and 17βHSD2 mRNA in the treatment group. No significant difference was observed in the amount of ERβ mRNA transcripts between the two groups of women, and although PR mRNA was reduced in the treatment group, the difference was also not significant.

Healthy fertile women were recruited as the control group. We acknowledge that women undergoing assisted conception are a different group with alterations in physiological steroid levels and possibly in the endometrium. Hence, recruiting untreated parous women as a control group may not be the

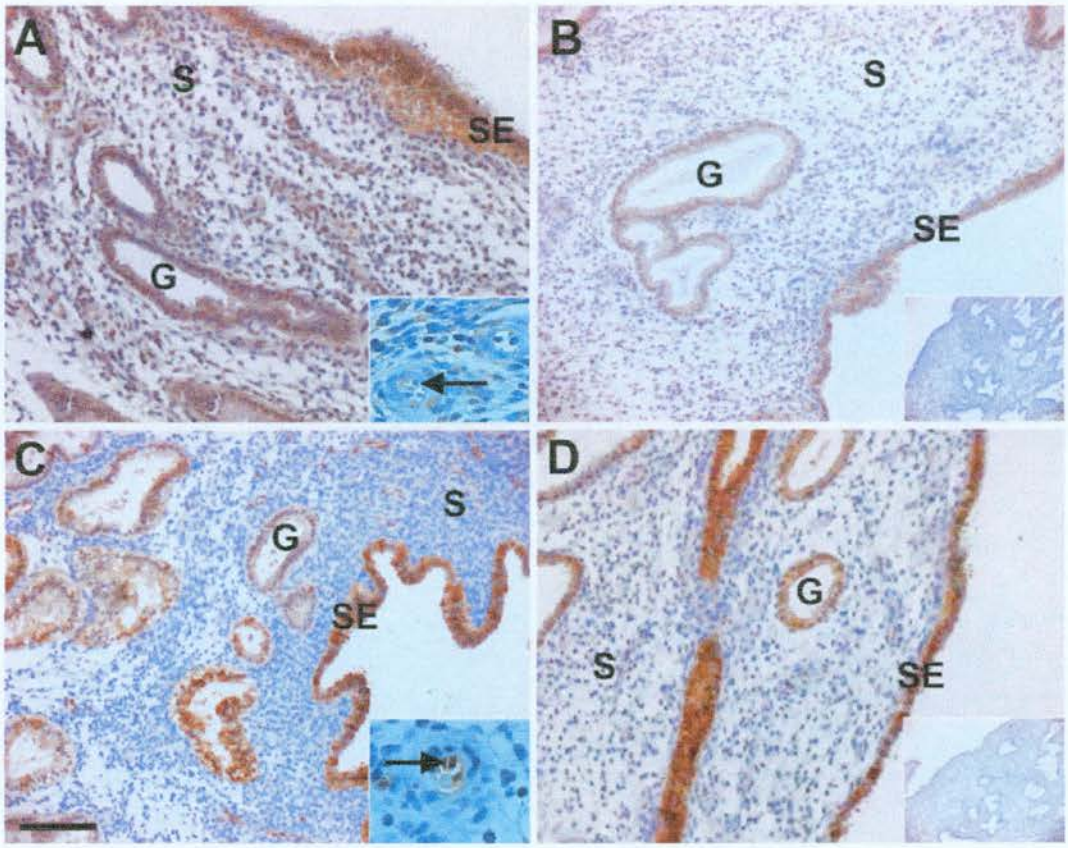

Figure 4: Immunohistochemical localization of selected steroid metabolizing enzymes in human endometrium of women in the mid-luteal phase of cycle (control group) and in women who have received rFSH and a GnRH antagonist (study group)
(**A**) 3βHSD immunostaining in endometrium from untreated women in mid-secretory phase—low level immunostaining at most cellular locations. (A) inset: low level immunostaining in vascular endothelium (arrow). (**B**) 3βHSD immunostaining in the endometrium from women treated with rFSH and GnRH antagonist—low level immunostaining at most cellular locations. (B) inset: 3βHSD negative—no immunostaining. (**C**) 17βHSD5 immunostaining in endometrium from untreated women in mid-secretory phase—moderate immunostaining in glands (G) and surface epithelium (SE). No immunostaining in stroma (S). (C) inset: moderate immunostaining in vascular endothelium (arrow). (**D**) 17βHSD5 immunostaining in endometrium from women treated with rFSH and GnRH antagonist—moderate immunostaining in glands and surface epithelium. No immunostaining in stroma. (D) inset: 17βHSD5 negative—no immunostaining. Scale bar = 20 microns

ideal equivalent comparison. However, our aim was to try and understand the differences between the physiological state of embryo implantation, which occurs in the mid-luteal phase in a natural ovulatory cycle, and in rFSH and a GnRH antagonist treated endometrium. Hence, it was appropriate to include untreated fertile ovulatory women in the control group.

In the subjects in the control group, an LH surge was detected using an alternate day urinary LH protocol. We acknowledge that urinary LH measurement may not be the most accurate way of checking the timing of ovulation. However, it is possible to identify the window of implantation on the basis of an alternate day urinary LH dating protocol. First, the window of implantation is thought to extend over Days 5–10 after the LH surge. Hence, even with urinary LH dating, we are likely to identify the putative window of implantation. Second, it is theoretically possible that the difference of 1 or 2 days could influence the result. We have however dated the endometrium not only by LH dating, but also by histological dating and mid-luteal serum progesterone concentration at the time of EB. We are thus confident that the endometrium was sampled during the mid-luteal stage and certainly during the window of implantation. There are no data to suggest that a difference of 1 or 2 days is likely to have a major impact on the sex-steroid receptor expression. Indeed with the exception of one study looking at the PR and ER expression at LH+7

Table 5: Summary of sex steroid and steroid metabolizing enzyme mRNA expression

	P-value
PR	0.12
ERα	0.02*[a]
ERβ	0.96
AR	0.01*[a]
3βHSD1	0.012*[a]
3βHSD2	Sample size too small
17βHSD2	0.028*[a]
17βHSD5	Sample size too small

*$P < 0.05$ denotes statistical significance (*t*-test after log transformation).
[a]indicates reduction of mRNA level in the study compared with control group.

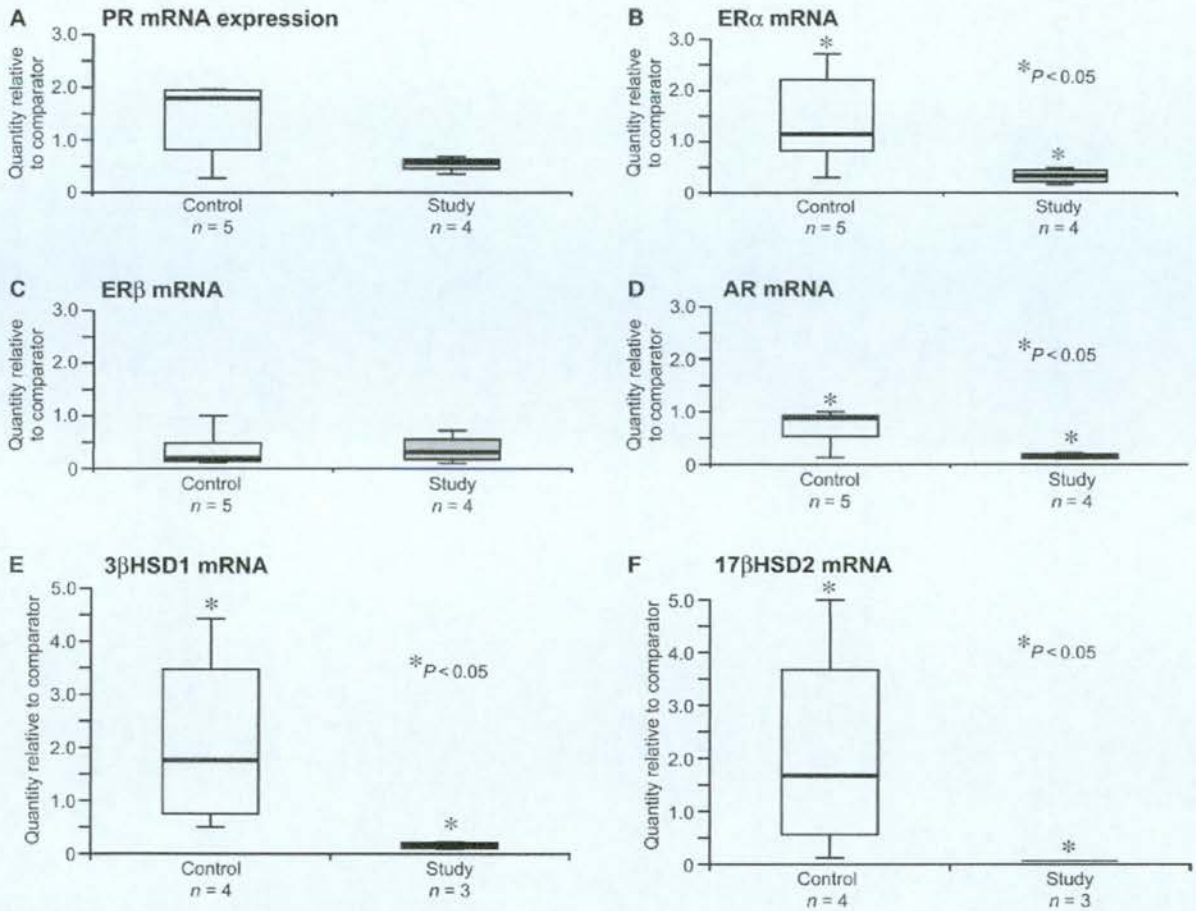

Figure 5: Quantitative evaluation of sex-steroid receptors and steroid receptor metabolizing enzymes mRNA expression of women in the mid-luteal phase of cycle (control group) and in women who have received COH and a GnRH antagonist (study group)
(**A**) PR, (**B**) ERα, (**C**) ERβ, (**D**) AR, (**E**) 3βHSD1 enzyme and (**F**) 17βHSD2 enzyme. All endometrial tissue samples were compared with an internal control (comparator) obtained during the mid-luteal phase of the menstrual cycle. ERα mRNA ($P = 0.02$) and AR mRNA ($P = 0.01$) levels were significantly reduced in endometrial tissue from women treated with a GnRH antagonist and rFSH. 3βHSD1 mRNA ($P = 0.01$) and 17βHSD2 mRNA ($P = 0.02$) levels were significantly reduced in the endometrial samples from women treated with a GnRH antagonist and rFSH. Note scale(Y axis) in E and F differs from A–D

after treatment with ganirelix (Simon *et al.*, 2005), where no alteration in the steroid receptor expression was reported, we are not aware of any data describing the steroid receptor expression in the mid-luteal phase after treatment with rFSH and a GnRH antagonist.

Luteal phase progesterone supplementation was administered to the oocyte donors (study group) to mimic an actual treatment cycle. Evidence exists to support the use of luteal phase progestogens after any form of down-regulation in an IVF/ICSI treatment cycle (Beckers *et al.*, 2003) and most centres offer this routinely. Administering progesterone supplementation in one group (study) and not in another (control) may add an element of bias. However, our aim was to compare the differences in the mid-luteal phase endometrium from fertile untreated subjects, i.e. the physiological state with the mid-luteal endometrium of women routinely treated with a GnRH antagonist and rFSH.

This study has shown that under the effect of a GnRH antagonist and rFSH with luteal phase progesterone supplementation, there is a significant down-regulation of PR in the surface epithelium. The observation of PR down-regulation in the surface epithelium is in agreement with other studies evaluating PR content in the secretory phase of a normal menstrual cycle. It has been suggested that under the influence of progesterone, PR declines in the epithelium at the beginning of the window of implantation (Lessey *et al.*, 1996). It therefore appears that any hormonal fluctuations that may result in the serum due to treatment with Cetrorelix and Gonal-F have little impact on intracellular PR in surface epithelium. In the secretory phase of a normal menstrual cycle, significant PR expression has been detected in the endometrial stroma (Lessey *et al.*, 1988). However, under the effect of a GnRH antagonist and rFSH and with progesterone supplementation, we found a significant down-regulation of PR in endometrial stroma. Several genes have been localized to the endometrial stroma and significant proportions are expressed during the secretory phase (Yanaihara *et al.*, 2004). Studies have also shown an important role for progesterone in the endometrial stromal cell in induction of extracellular matrix in relation to implantation (Nakamoto *et al.*, 2005). A significant

down-regulation of stromal PR could thus influence gene expression during the secretory phase and adversely impact endometrial receptivity. PR protein has not been identified in the vascular endothelium (Krikun *et al.*, 2005), but they are abundantly expressed in the perivascular cells throughout the menstrual cycle (Perrot-Applanat *et al.*, 1988; Critchley *et al.*, 2001). In this study, PR was significantly up-regulated in the perivascular cells of women treated with Cetrorelix and Gonal-F. Progesterone acting on the perivascular cells has been implicated in the modulation of endometrial blood flow. Furthermore, cytokine control in the perivascular cells is thought to be controlled by progesterone (Kelly *et al.*, 2002). These factors may well be important in the process of embryo implantation, and alterations of PR expression could influence these processes. PR expression in the glands of untreated women was variable. In regular cycling women, in the secretory phase, significant PR content was maintained in the stroma, but diminished in the glandular epithelium (Lessey *et al.*, 1988). In this study, in some control biopsies, moderate glandular PR expression was maintained however, as evident in the box and whisker plot, there was marked variability in expression between biopsies. The number of women included in our study was small and this may have contributed to the apparent trend towards an increased mid-secretory PR expression in glands in untreated women. Furthermore, the women included in the control group were different from those who provided the study biopsies in the treatment group. Whereas ideally, we would have liked to recruit the same oocyte donors as controls to further reduce the bias, practically it was only reasonable to seek an EB from an altruistic oocyte donor on a single occasion. In the control group, the mid-luteal phase was confirmed through consistency of reported LMP, circulating serum progesterone levels and histological dating. It is interesting to note that with rFSH and a GnRH antagonist treatment, glandular PR immunoexpression declined. This observation would be consistent with PR expression in the secretory phase of an untreated cycle. PR mRNA levels are known to vary during the human menstrual cycle. In the late proliferative phase, glandular PR mRNA levels are significantly higher but it reduced in the secretory phase. In the stroma, PR mRNA remains unchanged (Lau *et al.*, 1996). Although there was some reduction in PR mRNA levels in the treatment group compared with the controls, the difference was not significant. However, PR changes in whole biopsies may not reflect the subtle changes in steroid receptor expression that exist between individual cell types.

In regular cycling women, ERα and ERβ have been identified in the endometrial epithelium, stroma, glands and perivascular cells (Saunders and Critchley, 2002). Only ERβ and not ERα is expressed in the vascular endothelium (Critchley *et al.*, 2001). We did not observe any significant difference in immunoexpression of either ERα or ERβ in treated women. Controlled ovarian stimulation is known to lead to supraphysiological levels of E_2 and progesterone. These are thought to affect the endometrial receptivity through the predominantly progestational effects of endometrial phase advancement and premature luteinization (Kolb and Paulson, 1997). Hence, it appears that ER-mediated signalling appears to be of less importance in relation to implantation compared with PR mediated effects on the endometrium. It is interesting to note however that quantitative RT–PCR showed significantly reduced ERα mRNA levels in the treatment group.

AR expression is influenced by levels of circulating estrogens and androgens. Epithelial AR is up-regulated by estrogens and androgens and is inhibited by progestins (Slayden *et al.*, 2001; Apparao *et al.*, 2002). AR is expressed in the endometrial stromal cells. The intensity of expression declines from proliferative phase to mid-secretory phase. In late secretory phase, AR expression is diminished in all cell types (Mertens *et al.*, 2001). We found no significant difference in protein expression of AR in women treated with rFSH and a GnRH antagonist, however, QRT–PCR showed significantly reduced AR mRNA levels in the group of women treated with a GnRH antagonist and rFSH. So far, there are very limited data on effects of AR and AR-induced gene expression in humans. Studies in pigs show AR in the pig endometrium during the window of implantation and demonstrate the functional, albeit complex, interactions of androgens and estrogens in the regulation of uterine endometrial gene expression and cell growth *in vitro* (Kowalski *et al.*, 2004). Further studies are needed to evaluate AR-induced gene expression in humans and the potential impact on embryo implantation.

The 3βHSD is weakly expressed in the glandular epithelium of the proliferative phase and moderately expressed in the glandular epithelium of secretory phase of the endometrium (Rhee *et al.*, 2003). In this study, irrespective of presence or absence of treatment, immunoexpression of 3βHSD in the glands, surface epithelium and endothelium was at a low level. No immunoexpression was seen in stroma and perivascular cells. No significant difference was observed in endometrial 3βHSD immunoexpression between the two groups of subjects. This suggests that the pre-ovulatory supraphysiological levels of estrogen and progestogen that result from COH and use of GnRH antagonists do not lead to any significant alteration in the levels of 3βHSD protein during the window of implantation. The currently available antibody against 3βHSD recognizes both forms of human 3βHSD enzymes, types 1 and 2. Hence it is not possible to comment on changes in the amounts of 3βHSD1 protein. However, RNA studies indicate that 3βHSD1 mRNA transcripts may be changing. We observed a significant reduction in 3βHSD1 mRNA in women treated with a GnRH antagonist and rFSH. The 3βHSD1 is responsible for the conversion of inactive pregnenolone to active progesterone and of dehydroepiandrosterone to androstenedione. A reduction in 3βHSD1 transcripts will ultimately lead to a reduction of intracellular progesterone. In the presence of altered PR expression, as we observed with IHC, it is likely that the reduced ligand availability for binding to PR leads to a disturbance in the dynamics of ligand-receptor interaction. This may affect progesterone-mediated signaling pathways including alterations in gene expression profiles thereby affecting the receptivity of the endometrium.

The 17βHSD5 transforms androstenedione to testosterone and also progesterone to the inactive 20-hydroxyprogesterone, and in the endometrium its immunoexpression has been

localized to the surface epithelium and the vascular endothelium (Pelletier *et al.*, 1999). Our findings are in agreement with previous reports. We did observe moderate to intense 17βHSD5 immunoexpression in endometrial surface epithelium and vascular endothelium. However, in this study we also observed moderate 17βHSD5 immunoexpression in the endometrial glands. No immunoexpression was observed in stroma and negligible immunoexpression was observed in the perivascular cells. No significant difference was observed in the 17βHSD5 immunoexpression between the two groups of subjects. We observed a significant reduction of 17βHSD2 mRNA in women treated with rFSH and a GnRH antagonist. Since 17βHSD2 is involved in the inactivation of E_2 to estrone and converting androgens to less potent forms, it is likely that higher levels of intracellular E_2 and androgens persist thereby further disturbing the balance between estrogen, progesterone and androgens. This may further affect the endometrial development leading to suboptimal endometrial receptivity.

We have identified only one previous report comparing the effects of a GnRH antagonist treatment on mid-luteal phase endometrium to the state of the mid-luteal endometrium in a natural ovulatory cycle (Simon *et al.*, 2005). However, that study only examined ER and PR expression 7 days after an LH surge. The GnRH antagonist used in that study was Ganirelix. In this study, we used Cetrorelix and to our knowledge this is the first report describing the ER, PR and AR expression as well as the steroid metabolizing enzymes expression in a GnRH antagonist treated mid-luteal phase endometrium.

The reason, why so few studies have addressed the mechanisms of endometrial receptivity in IVF/ICSI cycles during the window of implantation is due to the difficulty in obtaining, for detailed studies, endometrial biopsies at this phase of the treatment. Hence, most of the relevant studies have been performed on only small numbers of subjects.

Conclusions

In summary, to our knowledge, this is the first report describing the effects of a GnRH antagonist and rFSH on sex-steroid receptor and steroid metabolizing enzymes expression in mid-secretory phase endometrium. This study has shown that under the effect of rFSH and a GnRH antagonist with progesterone supplementation, significant alterations occur in endometrial intracrinology at a time when the endometrium would be expected to be most receptive for implantation. The impact of these observations on embryo–endometrial interaction requires further evaluation.

Acknowledgements

We are grateful to all the women, especially the oocyte donors, who agreed to take part in this study. We would like to thank Teresa Henderson and Morag Hamilton for technical assistance, Ted Pinner for assistance with graphics and Sandra Brett for help with patient recruitment. We are most grateful to Trevor Penning for the gift of antibody against human 17βHSD5. Study supported in part by MRC Programme Grants G0000066 and G0500047 and the Edinburgh Assisted Conception Unit Endowment Fund.

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Submitted on February 22, 2007; resubmitted on May 23, 2007; accepted on July 11, 2007